PEPTIDE ENGINEERING FOR DEVELOPMENT OF ANTIMICROBIALS AGAINST *Mannheimia haemolytica*

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ABSTRACT

Mannheimia haemolytica (*M. haemolytica*)-induced bovine respiratory disease causes millions of dollars in economic losses to Canadian cattle industry. Contemporary management strategies built around the use of antimicrobials are proving to be increasingly unavailing and lead to drug residues in meat which may contribute to the development of multi drug resistant bacteria. Many *M. haemolytica* vaccines are effective in stimulating antibody responses but studies of vaccination in young calves and the cattle exposed to *M. haemolytica* (high-risk cattle) have shown poor vaccine efficacy. Antimicrobial peptides (AMPs) may help in the management of respiratory disease caused by *M. haemolytica* while minimizing the risk of drug residues in animal-derived food products.

AMPs are positively charged molecules that can kill bacteria primarily through the electrostatic interactions with the anionic bacterial lipid bilayer. Since the primary target of AMPs is the bacterial surface charge, which is evolutionarily conserved, the development of resistance towards AMPs seems less likely. These peptides hold potential to replace or reduce the use of antibiotics.

Human β-Defensin 3 (HBD3) and Microcin J25 (MccJ25) are cationic peptides that have shown good activity against many Gram-negative bacteria. Five peptides, namely native HBD3, three synthetic HBD3 analogues (28 amino acid, 20AA, and 10AA), and MccJ25 were selected for microbicidal activity against *M. haemolytica*. Three C-terminal analogues of HBD3 with all cysteines replaced with valines were manually synthesized using solid phase peptide synthesis (SPPS).

In the three analogues, replacement of cysteine with valine rendered them linear and increased their antibacterial activity. Minimum Bactericidal concentration (MBC) assays were performed

with the final inoculum size of 1-5x10^5 cells/ml, with the exception of the 10AA analogue which was incubated with 10^4 cells/ml final inoculum size. The antimicrobial assay showed that *M. haemolytica* was intermediately sensitive to HBD3, 28AA and 20AA analogue with an MBC of 50 µg/ml. MccJ25 had limited effect with an MBC greater than 100 µg/ml. The MBC value of 6.3 µg/ml achieved with the 10AA analogue is likely a result of lower final inoculum size.

AMPs have several immunomodulatory functions, and these peptides can act as chemoattractant, induce cytokine release that in turn leads to chemotaxis of monocytes and neutrophils. Since neutrophils play an important role in the pathogenesis of BRD, the chemotactic effect of HBD3, 20AA and 28AA peptides on bovine neutrophils was studied using Boyden chamber. Peripheral blood neutrophils isolated from normal healthy cattle showed chemotaxis towards HBD3 and 20AA peptides ($P<0.05$) but not towards 28AA analogue. Co-incubation of neutrophils with any of the peptides did not affect their chemotaxis towards N-formyl-L-methionyl-L-leucyl-phenylalanine (fMLP).

Based on these data, it can be concluded that HBD3 and its analogues showed antimicrobial effects against *M. haemolytica* but MccJ25 had limited microbicidal activity against *M. haemolytica*. While HBD3 and 20AA analogue were chemotactic for bovine peripheral blood neutrophils, none of the peptides inhibited fMLP-induced migration of neutrophils. These peptides hold potential for further *in vivo* testing to develop them for use to manage *M. haemolytica*-induced respiratory disease in cattle.

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LIST OF ABBREVIATIONS

AA: Amino Acid

AMPs: Antimicrobial Peptides

Boc: tert-butyloxycarbonyl

BRD: Bovine Respiratory Disease

DCM: Dichloromethane

DIC: 1, 3-diisopropylcarbodiimide

DIPEA: N,N-Diisopropylethylamine

DMF: N,N-dimethylformamide

DMSO: Dimethylsulfoxide

fMLP: N-formyl-L-methionyl-L-leucyl-phenylalanine

Fmoc: 9-Fluorenylmethoxycarbonyle

HBTU: O-Benzotriazole-N,N,N,N-tetramethyl-uronium hexafluorophosphate

HBD1: Human β-Defensin 1

HBD2: Human β-Defensin 2

HBD3: Human β-Defensin 3

HF: Hydrogen fluoride

HOBt: N-Hydroxybenzotriazole

HPLC: High Performance Liquid Chromatography

IL: Interleukin

LD50: Lethal Dose 50

LFA-1: lymphocyte function–associated antigen 1

LKT: Leukotoxin

LPS: Lipopolysaccharide

MALDI-TOF: Matrix-assisted laser desorption/ionization -Time of flight

MBC: Minimum Bactericidal Concentration

MccJ25:MicrocinJ25

mg: Milligram

M. haemolytica: *Mannheimia haemolytica*

MIC: Minimum Inhibitory Concentration

Min: Minute

mL: Milliliter

mM: Milimolar

MS: Mass spectrometry

NCCLS: National Committee for Clinical Laboratory Standards

NMM: N-methylmorpholine

OD: Optical density

PBS: Phosphate buffer saline

RNAP: RNA-polymerase

SPPS: Solid phase peptide synthesis

TFA: Trifluoroacetic acid

TFE: Trifluoroethanol

TNF-α: Tumor necrosis factor α

wt HBD3: Wild type Human β-Defensin 3

µg: Microgram

nM: Nano Molar

µL: Microliter

1. INTRODUCTION

1.1. Background

Canada's beef-producing industry and cattle sector are major contributors to the Canadian economy. According to Canada Beef Inc., Canada has nearly 83,000 cattle farms and ranches and this sector contributes upwards of $20 billion a year to Canadian economy. The beef and dairy cattle industry suffer significant economic losses due to *Mannheimia haemolytica* (*M. haemolytica*)-induced Bovine respiratory disease (BRD) [1]. *M. haemolytica,* a Gram-negative coccobacillus, which causes pneumonia in cattle, resides in the nasopharynx of cattle in a commensal relationship, but stress and viral infections compromise host defenses giving *M. haemolytica* an opportunity to penetrate lungs and cause infection [2]. A variety of vaccines and antibiotics against *M. haemolytica* are available but control and prevention is still a problem and due to which approximately $1 billion per year are lost by the US cattle industry [1-5] with similar relative losses the in Canada. Problems such as antibiotic resistance, antibiotic residue in meat and an inadequacy of vaccines push for the search of alternative treatments.

In this context, antimicrobial peptides (AMPs) have received significant attention as alternatives to antibiotics [6, 7]. Human β-Defensin 3 (HBD3) has been isolated from airways surface fluid from patients with psoriasis, suggesting it may play a role in fighting local infection [8]. Coupled with the fact that *M. haemolytica,* in normal condition, is prevented from entering into the lungs by the host's innate defense mechanism which includes the bovine defensins [2], it was thought that HBD3 peptide might be effective in *M. haemolytica*-induced pneumonia. Microcins are another class of AMPs which are produced by bacteria such as *E. coli*. Microcins inhibit the growth of many pathogenic Gram-negative bacteria with Minimum inhibitory concentration (MIC), in

the nanomolar range [7, 9-11]. Specifically, MicrocinJ25 (Mccj25) is a peptide comprised of 21 amino acids and is active against many Gram-negative bacteria such as *E. coli* and Salmonella. Mccj25 is stable in the presence of many proteolytic enzymes because of its unique lasso like structure [7]. Therefore, we hypothesize that HBD3 and Mccj25 might prove as potential candidates for application in the treatment of BRD. The objective of the current study was to design, synthesize, purify and evaluate the antimicrobial activity of HBD3 its analogues, and recombinant MccJ25 against *M. haemolytica*.

1.2. Pathophysiology of *Mannehemia haemolytica* induced Bovine Respiratory Disease

Multiple factors such as viral infections with bovine herpes virus-1, and stress caused by weaning and transportation may contribute to the progression of bovine respiratory disease [2, 5]. Although the mechanism by which susceptibility to *M. haemolytica* increases is still unclear some studies have suggested that during viral infections leukocyte function is altered [2, 4, 5, 12].

In healthy cattle the non-pathogenic serotypes of *M. haemolytica* S2 and S4 are most numerous, while pathogenic serotype S1 is low in number [13]. Factors such as weaning, mixing of cattle with various health conditions in the feedlot, transportation stress and micro-environmental changes induced by viral infections, may augment multiplication and colonization of the nasopharyngeal region by *M. haemolytica* serotype S1 [14]. Although the mechanism is not fully understood, BRD develops after S1 serotype preferentially colonizes the upper respiratory tract, where its further proliferation promotes its entry of into the trachea and lungs [14, 15].

M. haemolytica S1 evades the host immune system and colonizes lungs with the help of several virulence factors such as Capsule, adhesins, outer membrane proteins, lipopolysachharides

(LPS), leukotoxin (LKT), neuraminidase and glycoprotease [2, 3]. LKT and LPS are the most important virulence factors involved in the pathogenesis of BRD [2, 5, 15-17].

Leukotoxins derived from *M. haemolytica* play an important role in the development of pulmonary necrosis and pathogenesis of inflammation. Leukotoxin action in ruminants [18, 19] is induced through its interactions with the β2 integrin LFA-1 (lymphocyte function–associated antigen 1; CD11a/CD18) expressed on many host cells. Most cells express integrins, which play significant role in the intercellular communication and also as a communicator between a cell and extracellular matrix. Various leukocytes such as T lymphocytes, neutrophils, phagocytes and dendritic cells exclusively express β2 integrin, a subtype of integrin family [5, 20-24].

In vivo and *in vitro* results suggest that viral infections make the leucocytes more susceptible to the effects of *M. haemolytica* LKT by augmenting LFA-1 expression on bovine neutrophils and peripheral blood mononuclear cells via the release of mediators such as IL-1β, IFN-γ [4, 5] (Figure 1.1). Low concentration LKT triggers respiratory burst or degranulation in the neutrophils and macrophages. Proinflammatory mediators such as IL-1, IL-8 and TNFα are released from the activated macrophages and neutrophils, and histamine is released from mast cells. Higher concentrations of LKT or when leucocytes are exposed to a low to medium concentrations of LKT but for greater time period results in apoptosis, cell necrosis and osmotic lyses of the leucocytes [22, 25-27]. Activated neutrophils and macrophages release oxygen radicals, and they release their intracellular proteases following their necrosis exacerbates the existing inflammation [5, 28-30].

Although a major focus has been the role of alveolar macrophages in bovine respiratory disease, depletion of pulmonary intravascular macrophages attenuate lung inflammation due to reduced

expression of IL-8 and lesser accumulation of platelets in microvessels of lung [31]. Because of the complex interactions of viruses, bacteria and environmental factors, the pathophysiology of bovine respiratory disease is yet to be fully elucidated.

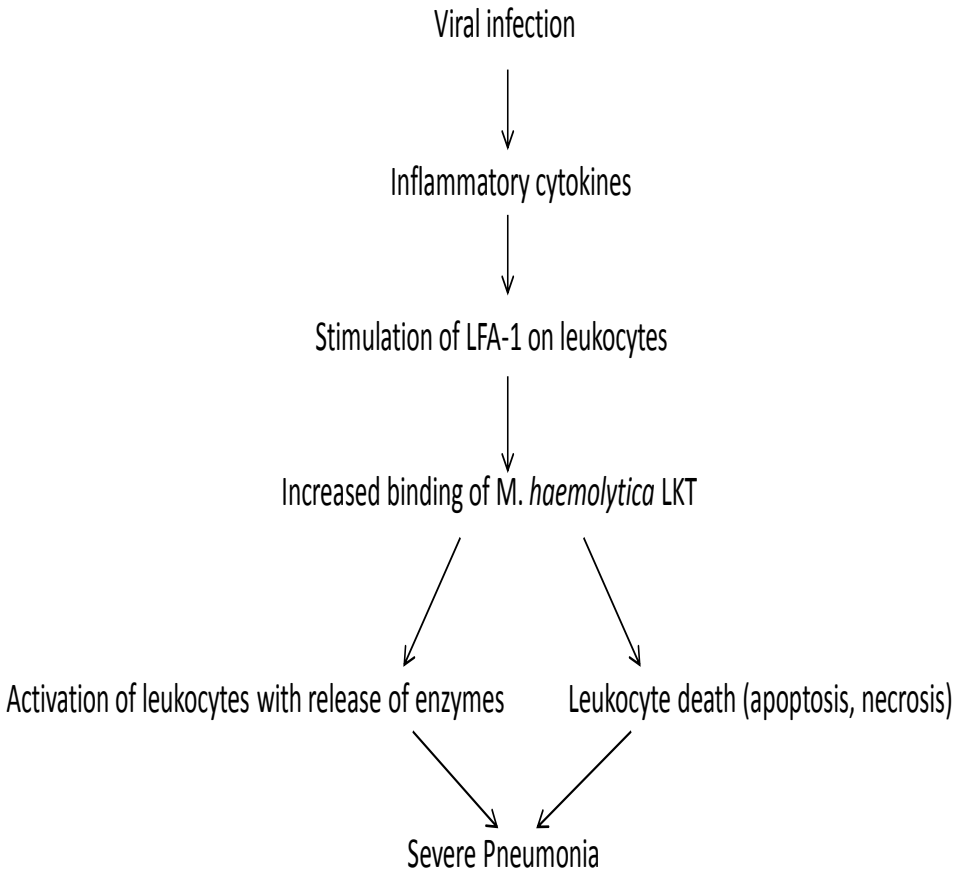


Figure 1.1. Working model of how viral infection might lead to increased susceptibility of bovine leukocytes to *M. haemolytica* [4].

1.3. Antimicrobial Peptides

Antimicrobial peptides (AMPs) play an indispensable role in innate immunity against various microbes such as fungi, viruses, Gram positive and Gram negative bacteria. These small cationic peptides have acted as the primary defense mechanism for most living organisms for over 2.6 billion years [32-34]. The primary mode of their action is disruption of bacterial cell membrane [35-43]. AMPs are positively charged and show a preference for bacterial membrane over mammalian cell membrane because bacteria are rich in anionic lipids such as phospahtidyl glycerol, have high transmembrane potential and do not possess cholesterol. Because they lack both net charge and cholesterol and also have a weaker transmembrane electric potential compared to bacterial cell membrane, mammalian cell membranes are relatively resistant to AMPs killing action [35-44] Two mechanisms of cell membrane disintegration have been proposed. 1) Barrel stave, accounts for a few peptides that are nonselective lytic peptides (lytic for both mammalian and bacteria cells) (Figure 1.2) [45-47]. 2) Carpet mechanism, is responsible for a wider category of peptides which are selective for bacteria (Figure 1.3), respectively [38].

The peptides that act through Barrel stave mechanism interact with the target membrane (inner cell membrane) via hydrophobic interactions. These peptides must form assortment of transmembrane pores by recognizing each other while in the membrane bound state and additional peptide monomers continue to associate with and increase size of the pore. While α-helical peptides must be approximately 22 AA in length, those with β-sheeted structure must be approx. 8AA long. Because transmembrane potentials of microbes is depleted by only a few pores, the peptides acting through Barrel stave mechanism are active at nanomolar concentrations [38].

Peptides working via the Carpet model do not self associate while membrane-bound. Most peptides act via the Carpet model which is suggested by the fact that many peptides with different length and amino acid composition act at the same molar concentration [38, 48-56].

Ever increasing bacterial resistance towards most of the prevalent antibiotics continues to provide impetus for the search of novel therapies. Although a plethora of new antibiotics such as β-lactams, glycopeptides, macrolides, ketolides, and aminoglycosides have been developed, the threat of increased bacterial resistance makes it imperative to continue the search for newer approaches and to supplement the existing drugs [57, 58].

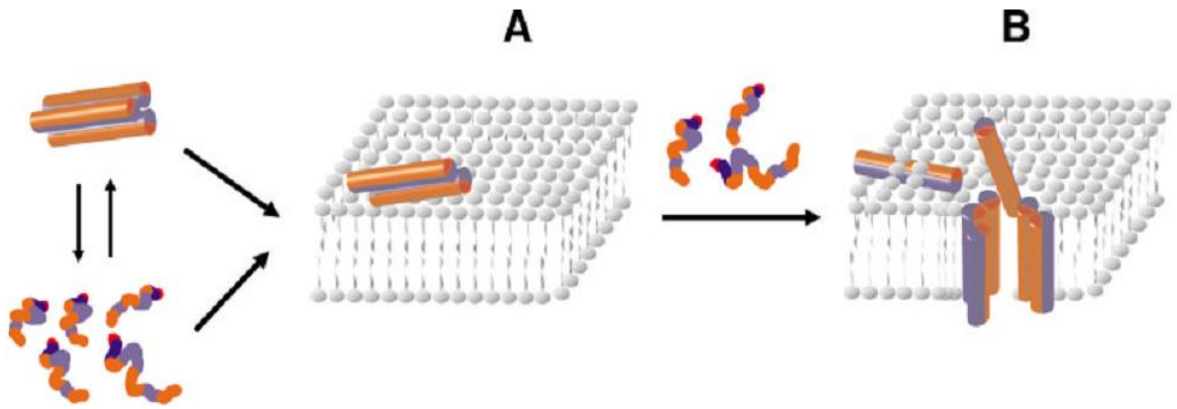


Figure 1.2. The barrel-stave model for pore formation in the membrane.

(A) Peptides monomers or oligomers assemble on the surface of the membrane (B), peptides enter lipid core of the membrane after employing more monomers."Blue, hydrophobic surface; red, hydrophilic surface" [38].

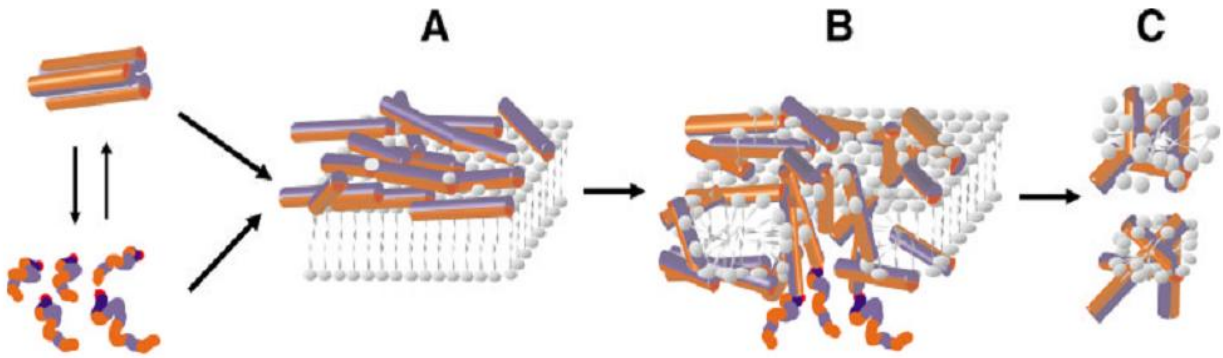


Figure 1.3. The proposed carpet model for membrane permeation.

(A) The positively charged peptides, in the form of monomers or oligomers, cover the membrane like a carpet such that their hydrophobic surfaces face the membrane and their hydrophilic surfaces are faced towards the solvent (B) Peptides reach a certain minimum concentration, infiltrate the membrane and form fleeting pores (C) Membrane is ruptured due to disruption of bilayer curvature. Blue: Hydrophobic surface; Red: Hydrophilic surface [38].

AMPs have a conserved three-dimensional structure, which may be critically required for anti-microbial activity [51, 57-61]. This common structure can be modified in a variety of ways to develop new AMPs for the cure and prevention of several ailments. Therapeutically, AMPs can be used alone or in synergy with other antibiotics or antiviral for enhanced effect [35]. AMPs can stimulate the immune system by inducing innate immunity and neutralizing endotoxins [38].

There are several peptides in Phase II and Phase III clinical trials and in the market, such as XMP.629, Omiganan (MBI-226) and Enfuvirtide. XMP.629 (Xoma LLC, Berkeley, CA, USA) were found to be active against *P. acnes* with MBC between 0.5 to 4.0 μg/ml. Topical dermal application of XMP.629 was found safe in a Phase I clinical trial, but a clear benefit was not found in a Phase II clinical trial involving 253 patients receiving topical therapy of acne. Omiganan (MBI-226) (Microbiologix Biotech, Vancouver, BC, Canada) obtained from bovine neutrophils is a cationic peptide proven to be a broad-spectrum antibiotic and antifungal agent *in vitro* [36, 43]. Omiganan was administered in a Phase III clinical trial to patients suffering from bloodstream infections. However, Omiganan did not produce a statistically significant reduction in the infection levels compared to povidone iodine (control) [43]. Enfuvirtide (INN) inhibits HIV fusion and is in clinical use for the treatment of HIV-1 infection in combination with other antiretroviral.

1.3.1. Neutrophils and Antimicrobial Peptides

Mammalian cell membrane is affected by AMPs in many ways. AMPs can affect the quicker resolution of infection and wound healing by stimulating cellular processes such as chemotaxis, angiogenesis and cytokine release. AMPs can also participate in antigen presentation.

AMPs such as LL37 can act in synergy with other peptides and enzymes such as lysozymes. LL37 in the presence of BD2 results in quick resolution of infection. AMPs are active *in vivo* under normal conditions at much lower MIC than when tested *in vitro*. One such example is LL37, which has a MIC greater than 32 μg/ml against *E. coli in vitro* but the *in vivo* active concentration of LL37 is less than 2 μg/ml.

There is a growing body of evidence indicating that AMPs act through indirect mechanisms to provide protection such as through stimulation of chemokine or cytokine production or influencing gene expression in the host. For example, AMP can inhibit pro-inflammatory cytokine production induced by endotoxin, promote chemokine release and modulate the responses of dendritic cells or T cells (Figure 1.4) [62]. The chemokine production induced by AMPs can cause recruitment of new inflammatory cells to the site of infection.

Human neutrophil peptide 1 (HNP-1) and HNP-2 are α-defensins that promote the recruitment of monocytes to inflammatory sites through their direct or indirect chemotactic activity [63]. Similar to α-defensin AMPs, HBD3 and HBD4 are chemotactic for monocytes and macrophages [64]. AMPs such as HBD3 recruit leukocytes directly as well as through induction of expression of chemokines or cytokines such as CXCL8 (IL-8) and CCL2 (monocyte chemoattractant protein, MCP-1).

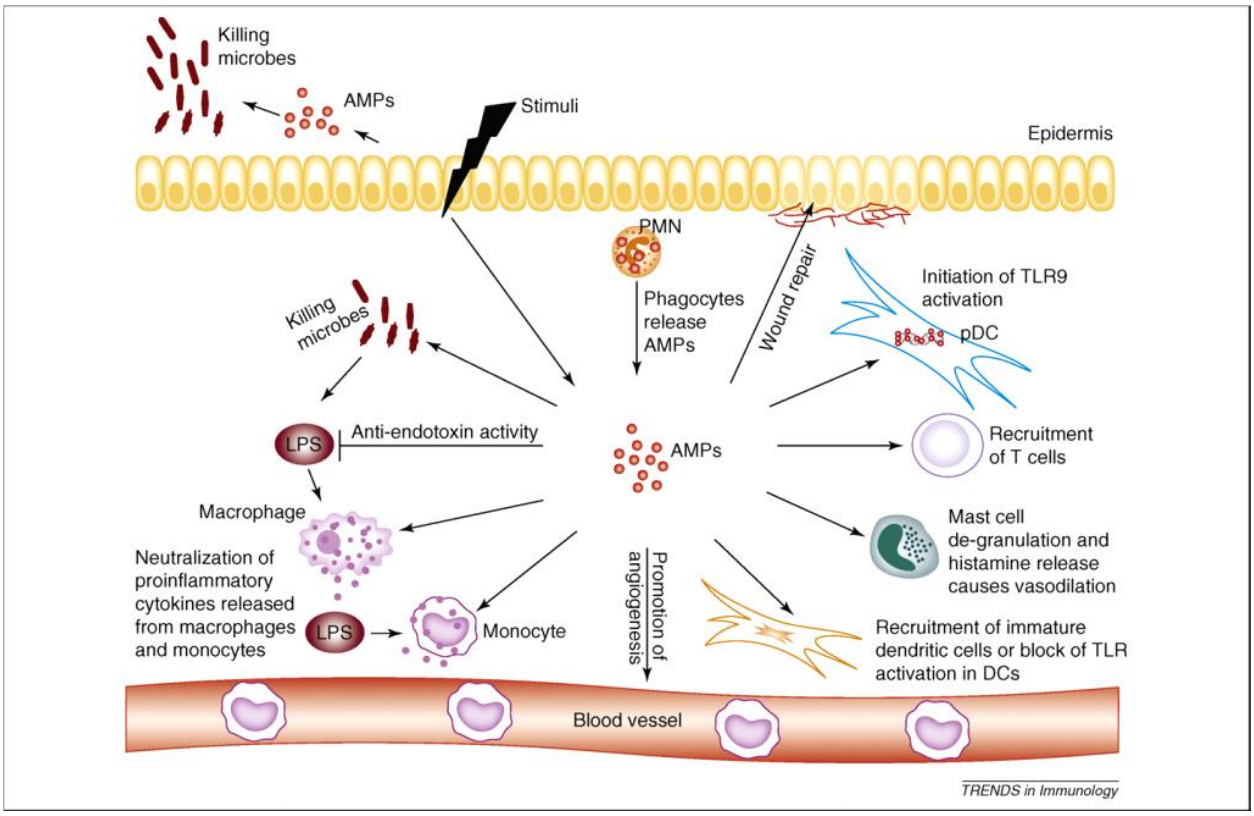


Figure 1.4. Multiple functions of antimicrobial peptides in host defense.

AMPs induce a variety of responses in monocytes, macrophages, neutrophils and epithelial cells. They alter gene expression of host cells, induce production of chemokines and cytokines, promote leukocyte recruitment to the site of infection, influence cell differentiation and activation and block or activate innate immune responses to induce protection against infection, selective control of inflammation, promotion of wound healing and initiation of adaptive immune responses. Abbreviations: AMP, anti-microbial peptide; DC, dendritic cell; LPS, lipopolysaccharide; pDC, plasmacytoid dendritic cell; PMN, polymorphonuclear cells; TLR, Toll-like receptor [62].

1.3.2. HBD3, HBD3 analogues, and Microcin J25 (MCCJ25)

HBD3 is a cationic peptide, highly basic with a high positive charge (+11) [65] and consists of 45-amino-acids (Figure 1.5 & Figure 1.6). HBD3 has a broad spectrum antimicrobial activity and it may also act as a chemoattractant molecule. It is active against many Gram-negative and Gram-positive bacteria. Its antimicrobial activity not only depends upon the overall hydrophobicity and charge but, also on their overall distribution [66]. HBD3 has disulfide bonds that are responsible for its chemotactic property; disulfide bonds promote chemotaxis by activating the chemotaxis receptors and by increased binding. However, HBD3 disulfide bonds do not have any antimicrobial function. HBD3 kill bacteria by causing perturbations in bacterial membrane, via electrostatic interactions with negatively charged bacterial cell membrane [67].

HBD3 does not require disulfide bridges to mediate its antimicrobial activities, however they responsible for proinflammatory chemotactic properties and therefore may contribute to side-effects [65]. The emergence of resistance against HBD3 seems not very likely since it acts primarily by the electrostatic interaction with the oppositely charged membrane of microbes. Thus development of resistance to anti-microbial peptides would require microbes to change their surface charge, which is evolutionarily conserved and not likely to occur [67, 68].

Wild-type HBD3 and three peptide fragments derived from the C-terminal are used in this study. In the three C-terminal analogues, namely 10 amino acid (AA), 20 AA, and 30 AA, cysteine (Cys) residues from the wild type sequence were substituted with Valine. It has been shown that substitution of the Cys residues with Tyrosine, Tryptophan or Valine retained potent

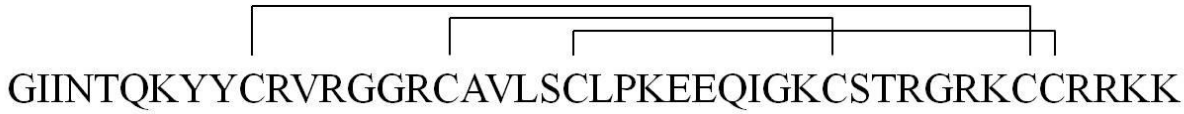


Figure 1.5. Amino acid sequence of HBD3. The three disulfide bonds between cysteine residues are shown.

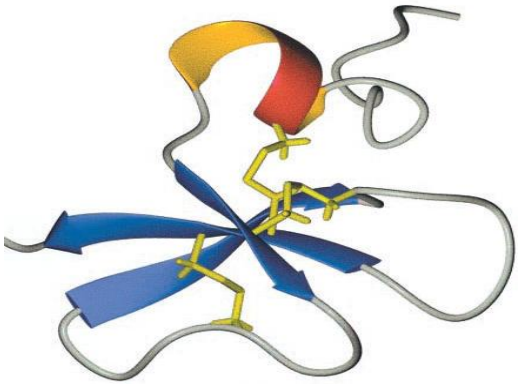


Figure 1.6. Ribbon diagrams of the three human β-defensin3.The three-disulfide bonds shown in gold [68].

GGAGHPEYFVGIGTPISFYG

Figure 1.7 Amino acid sequence of MccJ25. Amino acids 1 and 8 connect to form a ring.

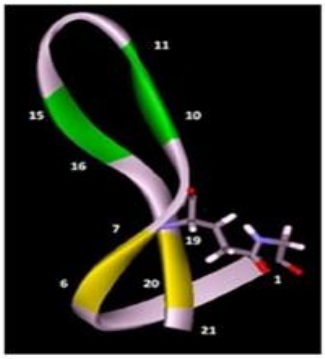


Figure 1.8. The ribbon representation of the lasso structure of MccJ25 is shown [70, 71].

anti-bacterial activity against *Pseudomonas aeruginosa*, a Gram-negative bacteria implicated in eye infections [66]. In addition, flexible analogues resulting from the removal of disulfide bridges have shown reduced cytotoxicity towards host cells [65].

Microcin are antibiotics produced by many strains of *E. coli* to secure nutrients from other bacteria in its vicinity. *E. coli* has specialized pumps to quickly secrete it. The 21 residue Microcin J25 is one of the microcin families; it has a "lassoed tail" structure, which makes it extremely stable to many proteolytic and metabolic enzymes. (Figure 1.8) [69]. Out of 21 amino acids, 8 amino acids form a ring. The ring is formed by the formation of an isopeptide bond between the free N-terminal amine group and amino acid residue glutamic acid which occupies the eighth position in the sequence [70, 71]. The remaining 13 amino acids form a loop which passes through the ring. Amino acids phenylalanine and tyrosine occupy 19 and 20th place in the sequence and face each other to maintain the loop [7, 9-11].

MccJ25 acts by two different mechanisms. First, it primarily inhibits transcription by obstructing the secondary channels of the RNA-polymerase (RNAP) and as a result prevents the access of the substrates to its active sites [69, 72-74]. The second mechanism of action is disrupting the electric potential of the target cells. MccJ25 has also been shown to inhibit oxygen consumption and the respiratory chain enzymes in E. coli [73].

1.4. Solid Phase Peptide Synthesis

SPPS is a general method of building an amino acid (AA) chain on an insoluble resin which is inert to the solvents used in the process. First, amino acid is loaded on the solid support contained in a syringe (Figure 1.9). All amino acids have their alpha amino group and side chain protected by groups such as tertiary-butyloxycarbonyl (Boc) or 9-fluorenylmethyloxycarbonyl

(Fmoc) to prevent undesired amino acid polymerization [75] [76]. After adding first AA to the resin beads, the alpha amino group of the first amino acid, which is masked by fmoc, is deprotected and the resin is washed with the solvents such as DCM and DMF. The next amino acid is acylated (i.e. activated) and then coupled with the amino acid attached to the resin support. The whole process of deprotection and coupling is repeated until the desired peptide length is obtained. At the end, the peptide chain is cleaved from the resin support in a process called cleaving. All side chain protecting groups are also simultaneously cleaved in the process [77]. (An overview of SPPS process is shown in Figure 1.10)

SPPS is carried out either by Boc or by Fmoc chemistry. Boc and Fmoc are protecting groups that protect Nα amino group. In Boc SPPS, the Boc group is deprotected by trifluoroacetic acid (TFA) and a tertiary amine is required to neutralize the free amino acid that is in the form of a TFA salt. Hydrogen fluoride (HF), which is a stronger acid than TFA, is used to cleave peptide from the resin; side chain protections are simultaneously cleaved. However, Boc chemistry has several disadvantages such as the repeated TFA acid treatment is deleterious as it causes unwanted side reactions, HF treatment may be too harsh for some fragile sequences and HF is potentially a very hazardous chemical.

Fmoc SPPS utilizes an orthogonal concept, which means the Nα amino and side chain protecting groups are detached by different mechanisms because they are different chemical entities [71, 76, 78]. The Nα amino group is protected by Fmoc and is removed in basic conditions, by piperidine in N, N-dimethylformamide (DMF), whereas side chain protecting groups are simultaneously cleaved at the time of peptide cleavage, in acidic conditions, by TFA. The milder reaction conditions make Fmoc synthesis preferable over Boc chemistry [75].

1.4.1. Resin Support.

Polymer matrix or resin support plays an important role in the success of SPPS. Polystyrene resin cross-linked with 1% of 1,3-divinylbenzene is a commonly used polymer matrix. This resin swells 2-6 fold when treated with DMF or dichloromethane (DCM), which is desirable characteristic as it increases the rate of diffusion and increase the kinetics the of reaction. Greater swelling facilitates greater diffusion of the reagents into the matrix [78, 79].

A linker connects resin beads to the carboxyl group of the first amino acid. There are a variety of resins such as Rink, Wang, and their derivatives but we chose to use an acid labile resin called 2-Chlorotrityl chloride resin (Figure 1.11) which is suitable for Fmoc-SPPS. The steric bulk of 2-Chlorotrityl chloride resin prevents diketopiperazide formation when the second amino acid is loaded [80].

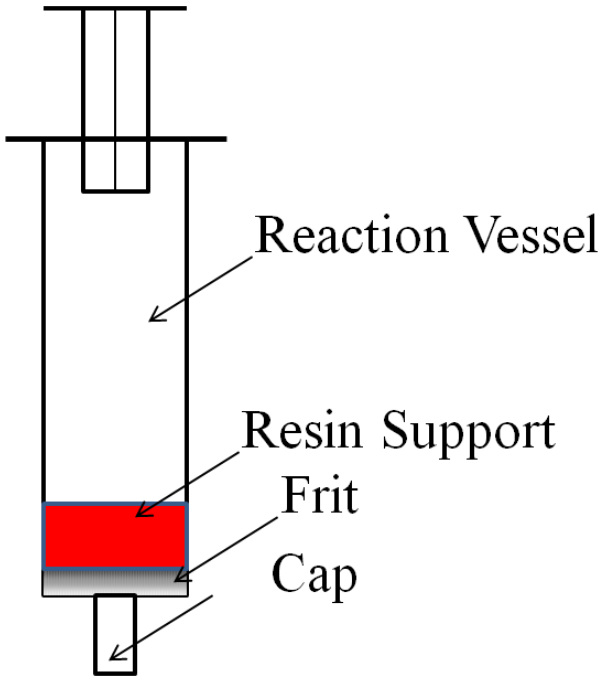


Figure 1.9. Syringe like reaction vessel for manual SPPS.

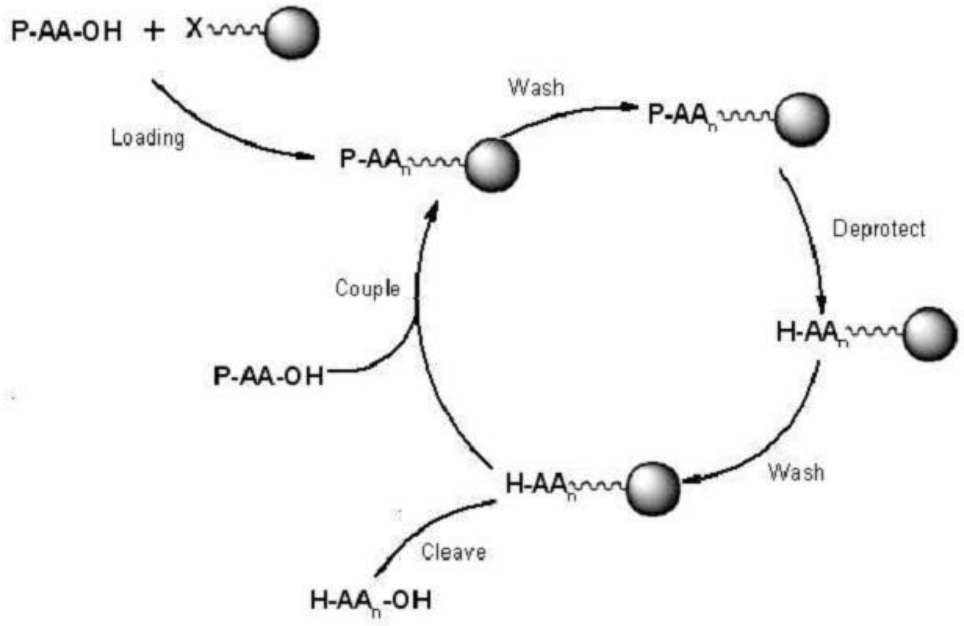


Figure 1.10. Normal SPPS cycle, P-AA-OH represent Fmoc protected amino acid and H-AA-OH symbolize amino acid with a free amine [77].

1.4.2. AA Coupling

Coupling reaction means a peptide bond formation between two α-amino acids. A peptide bond also known as an amide bond is a bond between carboxyl group of one AA with the α-amino group of another. Normally a reaction between an amine with a carboxylic acid results in the formation of a stable salt (Scheme 1). Hence, the carboxyl group needs to be acylated i.e. activated by introduction of a leaving group 'X' and production of carboxylic acid derivatives (RCOX). 'X' is an electron withdrawing group which increases the susceptibility of the carbonyl carbon to nucleophilic attack by an amino group (Scheme 2) [81].

Coupling reagents are used to increase yield and decrease racemization, which may occur during amide bond formation [81]. In our coupling reaction mixture we used (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate) for acylation of carboxyl to give active ester while hydroxybenzotriazole (HOBt) is used to prevent racemisation and a hindered or a non-nucleophilic base NMM.

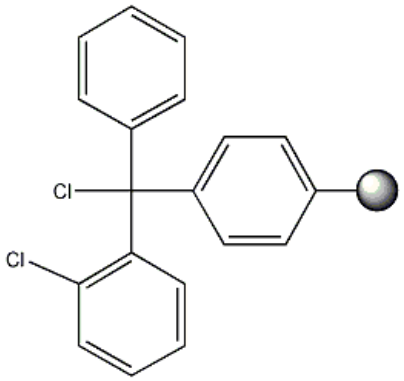
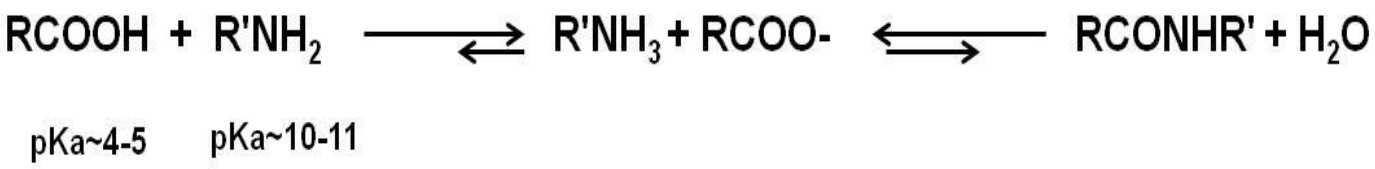


Figure 1.11. 2- Chlorotrityl resin.

Scheme 1: Amide bond formation [82].



Scheme 2: Activation of acid and amide bond formation [82].

1.4.3. Coupling Monitoring

A Kaiser or ninhydrin test is a qualitative test that is sensitive to primary amines and is used to detect completion of coupling reaction. In this test, few beads of resin are taken from reaction vessel and mixed with 2 drops of each ninhydrin, phenol, and pyridine in a test tube. Then, this solution is heated for 3 min at 110 °C and color change is observed. Blue/purple resin beads are indicative of uncoupled amines. Pale yellow/brown color is a confirmation of completion of coupling [77].

1.4.4. Peptide Cleavage

Trifluoroacetic acid (TFA) causes acidic conditions in the cleavage mixture lead to the release of the peptide in the free acid form [82]. The resin beads are shaken for about 60 minutes with a solution of TFA and water 95:5. Then, the syringe is drained and the filtrate collected into a glass flask. Cold diethyl ether (Et_2O) is then added to precipitate the crude peptide mixture. After precipitation peptide is then filtered and subsequently washed with cold Et_2O. Liquid in the crude peptide mixture is then evaporated on rotavapor and the crude peptide mixture is dissolved and lyophilized. MALDI-TOF analysis is then performed on the lyophilized peptide.

1.4.5. Purification and identification of peptides

Reversed phase high performance liquid chromatography (RP-HPLC) is used for purifying crude peptides because it can separate peptides with very little difference in amino acid sequences. Various components in crude peptide mixture elute at different time and give a peak on the chromatogram and the substance eluted at each peak were tested by MALDI-TOF mass analysis. The peak which has the desired molecular mass is identified and that fraction is collected to obtain the desired peptide.

1.4.6. Mechanism of purification

RP-HPLC purifies a peptide based on the principle of differential partitioning, peptides partition between the polar mobile phase, and the hydrophobic stationary phase. Small peptides get partitioned as well as adsorbed on the stationary phase, but polypeptides are only adsorbed on the stationary phase [83] (Figure 1.12). When the concentration of organic modifier (e.g. acetonitrile) reaches a critical range, the peptide desorbs. This swiftness of desorption is what makes RP-HPLC competent in separating peptides with very slight differences. However, in the case of small molecules desorption could occur slowly because, they along with being adsorbed, are also partitioned in the hydrophobic phase [83-85] (Figure 1.13).

When performing Gradient elution, the concentration of nonpolar mobile phase is increased gradually over time. For example, organic modifier concentration is changed from 20% Acetonitrile (Acn) in water to 50% Acn in water in 60 minutes. This elution method helps in achieving precise organic solvent concentration required for a particular molecule [83].

1.4.7. Identification of peptides

To calculate mass of peptide analogue, the software PAWS calculates mass based on the amino acid sequence provided. MALDI-TOF analysis was used to detect molecular weight of the synthetic analog at various time points during synthesis, to ensure proper chain elongation, and after completion of synthesis.

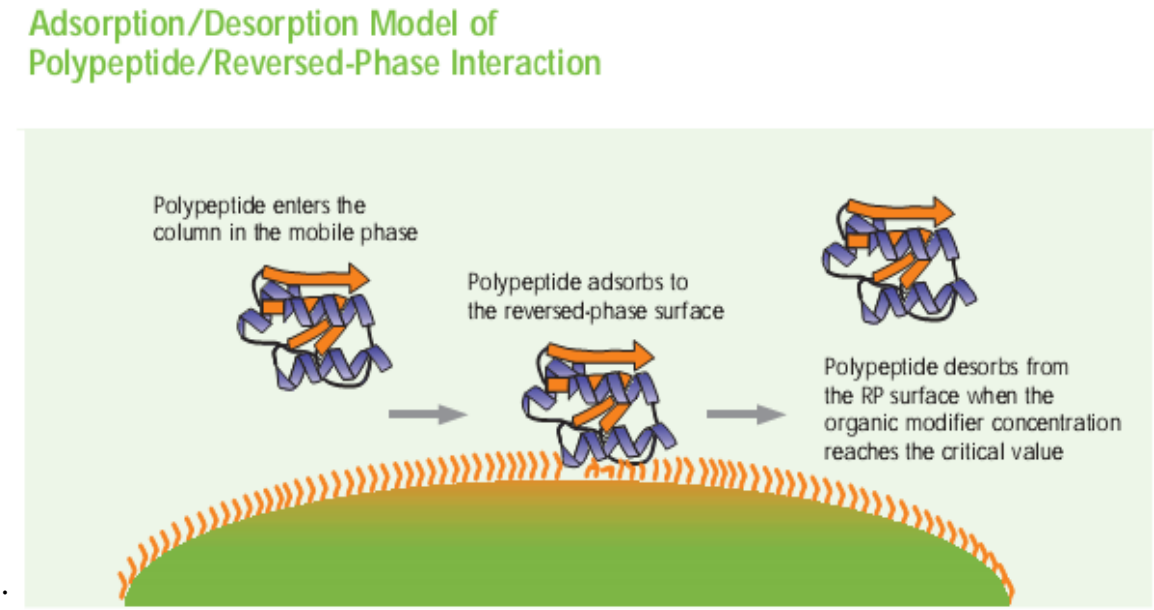


Figure 1.12. Polypeptide in the mobile phase introduced in to the column.

The hydrophobic region of the polypeptide attaches via adsorption to the surface of hydrophobic stationary phase and stays there until the concentration of organic solvent increases to the critical level and detaches the polypeptide [85].

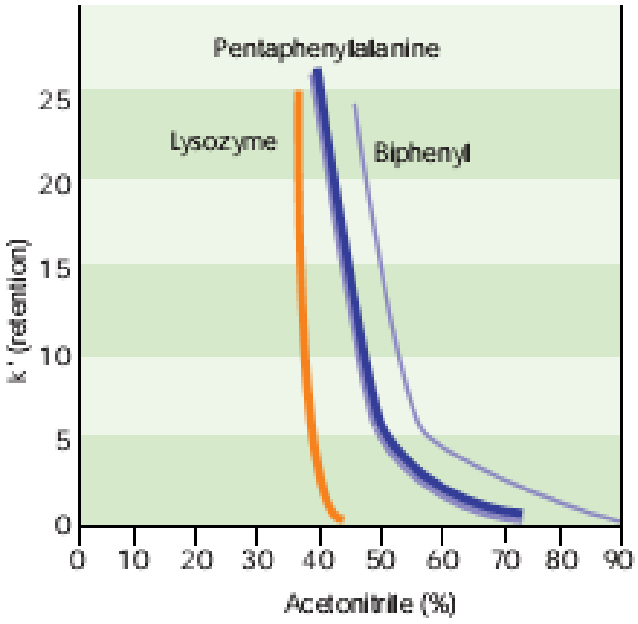


Figure 1.13. The retention time (total time during which a peptide remain attached to the stationary phase) of large peptides is faster than that of a small peptide.

Lysozyme desorption is most quick followed by Pentaphenylalanine, a small peptide, where as biphenyl, a small molecule, desorbs most gradually [85].

2. HYPOTHESES

a. Antimicrobial peptides, HBD3, HBD3 analogues and MicrocinJ25 are bactericidal against *M. haemolytica*

b. Unlike wtHBD3, the HBD3analogues and MicrocinJ25 will not alter functioning of neutrophils

3. OBJECTIVES

a. To synthesize, purify and characterize analogues of HBD3 as well as over-express MicrocinJ25 (MccJ25) in *E. coli*

b. To test the antimicrobial activity of native HBD3, synthetic HBD3 analogues, and MccJ25 against *M. haemolytica*

c. To study the effect of native HBD3, synthetic HBD3 analogues, and MccJ25 on normal neutrophils

4. RATIONALE

There is a need to develop newer antimicrobial compounds because of the concerns of antibiotic residues in cattle derived food products, development of antimicrobial resistance and lack of effective therapeutic and prophylactic compounds against *M. haemolytica* induced lung inflammation.

We propose to design and synthesize antimicrobial compounds belonging to Microcin and human β-defensin3 and to evaluate their antimicrobial activity against *M. haemolytica, in vitro*. Because neutrophils are central to host defense against bacteria, it is important that the new antimicrobial compounds do not have deleterious effects on the anti-microbial activities of neutrophils. Therefore, we will also evaluate the effects of synthesized antimicrobial molecules on neutrophils.

5. MATERIALS AND METHODS:

5.1. Expression and Purification of Microcin J25

Wild-type Microcin J25 (MccJ25) was used in this study as formation of the lasso ring is not possible synthetically. The peptide was expressed using a high copy number plasmid pTUC202 (a gift from Rutgers University USA), which carries the MccJ25 biosynthetic gene cluster, in competent *E. coli* MC4100 cells. It was grown in 2L M9 minimal media for 18 h at 37^0C. The culture supernatant was obtained by centrifugation at 4000 *g* for 15 minutes, and then subjected to two successive purification steps. First, it was applied on the flex column filled with XAD16 resin (Aldrich, CA). Two successive elution steps were performed with (30:70, v/v) and (80:20, v/v) methanol/water mixtures. MccJ25 is eluted in the (80:20, v/v) methanol/water mixture. MccJ25 was purified using reversed-phase HPLC (Varian Prostar 210, U.S). The purity of the peptides was confirmed by MALDI-TOF mass spectrometry.

5.2. Synthesis and Purification of HBD3 Analogues

HBD3 fragments were synthesized manually on 2-chlorotrityl chloride resin (0.2 mmol, 1 mmol/g). The first amino acid was coupled using DIPEA for 5 hours. Further amino acids were coupled at 2-fold excess using HCTU/HOBT/NMM as activating mixture in DMF. Amino acid coupling was performed for 3 hours at room temperature to ensure the completeness of the reaction, followed by Fmoc group deprotection using piperidine in DMF. These two reactions were repeated until assembly of the peptide was complete. A Kaiser test was performed after coupling of each amino acid. After completion of the synthesis, peptides were cleaved from the resin and all protecting groups were removed using cleavage mixture at room temperature. Cleaved peptide was concentrated by rotary evaporation. Cold ether was added to precipitate the peptide and

crude peptide was isolated after centrifugation. Crude peptides were dissolved in water and purified using reversed-phase HPLC (Varian Prostar). The purity of the peptides was confirmed by MALDI-TOF mass spectrometry.

5.3. Antimicrobial activity testing of peptides

Wild-type HBD3, its analogues, and MccJ25 were tested against *M. haemolytica* using optical density method and colony count assay. Briefly, an aliquot (5 µl) from bacteria suspension stored at -80^{o}C was put in 5 ml BHI broth at 37^{o}C for 18 hours. An aliquot (10 µl) from this overnight culture was added to fresh BHI broth and incubated at 37^{o}C for a further 5 hours to obtain mid-log phase bacteria. The culture was then centrifuged for 10 min at 800 *g* at 4^{0}C. The supernatant was discarded and the bacterial pellet was resuspended in cold, sterile sodium phosphate buffer (SPB) and washed again at 800 *g* for 10 minutes at 4^{o}C. The pellet was resuspended in 5ml SPB. To calculate the number of CFU/ml in the 5 hour culture, six successive 10 fold dilutions were made. From the last dilution 100 µl was plated in duplicate on petri dishes containing BHI agar and incubated for 24 hours. After 24 hours the colonies were counted in the two petri dishes and then averaged. Total number of colonies was the average number of colonies multiplied by the dilution factor.

All assays were carried out in sterile 96-well polypropylene flat-bottomed plates using a broth microdilution method. Two-fold serial dilutions of peptide were made in SPB, and 50 µl of each concentration of peptide was added to the wells of the assay plate. Fifty µl (25000 cells) of the bacterial suspension adjusted to 5x10^{5} cfu/ml in incubation media was prepared by adding 200 µl of BHI broth in 6.8 ml SPB and added to each well. Positive control wells contained 50 µl of the bacteria with 50 µl of 64 µg/ml ampicillin and negative control wells contained 50 µl of SPB and

50 µl bacterial suspension. Sterility control well contained 100 µl BHI broth to test and ensure that broth was not contaminated (Figure 5.1)

The plates were sealed with aluminum foil and were incubated at 37^{o}C for 2 hours then 50 µl of the contents of the well were pipetted on BHI agar plates and incubated for 24 hours. The bactericidal activity was expressed as the MBC which is the concentration at which 99.9% of the colonies are killed and the Lethal dose 50 (LD50) at which 50% or more bacteria are killed [51-53].

100.0 50.0 25.0 12.5 6.3 3.1 1.6 0.8 µg/ml

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	B	B	B	B	B	B		NC	SC	PC
B	B	B	B	B	B	B	B	B		NC	SC	PC
C												
D												
E												
F												
G												
H												

Figure 5.1. Layout of the setup of a polypropylene 96 well plate for antimicrobial susceptibility testing.

The doubling dilutions (100 µg/ml to 0.8 µg/ml) of an antimicrobial peptide were incubated with the peptide. B: Bacteria (*M. haemolytica)* and peptide incubated together in one well; NC: Negative control (*M. haemolytica* only); SC: Sterility control (broth only); PC: Positive control (*M. haemolytica* with ampicillin).

5.4. NEUTROPHIL ISOLATION AND CHEMOTAXIS ASSAY

5.4.1. Neutrophil Isolation

Neutrophils were isolated from cattle using established methods [86]. For each individual animal, blood was pooled into 50 mL tubes and diluted with an equal volume of PBS then 12.5 mL of the diluted blood was layered over 10 mL of Ficoll Paque PLUS while taking care to preserve the interface between the two liquids. Following centrifugation at 400 *g* for 30 minutes at 20^{o}C with the brake turned off, the lymphocyte layer was discarded along with Ficoll and plasma. The pellet was washed in 20 ml PBS with centrifugation at 500 *g* with low brake for 10 minutes. The supernatant was discarded and the pellet was suspended in equal volume of sterile water and gently mixed for 20 seconds followed by addition of equal volume of 1.8% NaCl to restore tonicity. The mixture was centrifuged at 500 *g* for 10 minutes and followed by another washing and centrifugation at 4^{0}C. The resulting neutrophil pellet was resuspended in PBS. Bovine neutrophils, collected in the above manner had more than 92% purity, and their viability was greater than 95% based on Trypan blue exclusion assay.

5.4.2. Chemotaxis assay

fMLP (114 nm) was used as the chemoattractant. Approximately 25 µl of peptides (50 µg/ml) was put into lower compartment wells of a 48-well Boyden Chamber. After the fMLP loading, the polycarbonate membrane filter (pore size 5 µM), with the shiny side up, was placed over the lower chamber. Next, silicone gasket was placed on the membrane, then the upper chamber was put on top of the gasket and the lug nuts firmly secured. Cell suspension (1 X 10^{6} cells) was then placed over control wells and N/P wells (neutrophil at top and peptide in the bottom wells). The whole assembly was then incubated at 37°C in humidified air with 5% CO_2 for 20 minutes. After

the incubation, the Boyden chamber was disassembled and the membrane carefully held with the bulldog clamp and the cells on the upper shiny surface scraped with the wiper blade. Next, the filter membrane was stained with the Diff-Quick and mounted on glass slide with the bottom side up. The cells within the filter pores were then counted in 5 random fields under light microscope at 40 X. The results are presented as the number of migrated neutrophils per microscopic field.

5.4.3. Migration inhibition Assay

Since peptides can be immunomodulatory, migration inhibition was carried out to determine if the peptides inhibit neutrophil migration. This assay was performed in the same manner as the chemotaxis assay with the only difference being that peptide and neutrophils were incubated together on the top of the filter; 25 µl of 100 µg/ml peptide and 25 µl (2 X 10^6 cells) of neutrophils were put together. In the lower wells 25 µl of 114 nM fMLP (chemoattractant) was placed followed by the staining and counting of the cells as described above.

5.5. Statistical Analysis

Data from susceptibility testing assay was analyzed using the non-parametric statistics (using ranked data), because the data were variable among groups most likely due to small sample size. The comparisons were done using contrasts (1 compared to 2, 2 compared to 3, and so on), given that we expected a decrease in colony count with increasing concentrations. Groups were considered statistically significant if the P-value was less than 0.05. A One-way ANOVA followed by Dunnets Multiple Comparisons test was performed on data obtained from the chemotaxis and migration inhibition assay. Significance was recorded when $P<0.05$. Computer software GraphPad Prism 6 and SPSS for migration and susceptibly assays, respectively, were used.

6. RESULTS

6.1. Peptide Design and Synthesis

Five peptides, Microcin J25 (MccJ25), HBD3, 10AA HBD3, 20AA HBD3 and 28AA HBD3 were evaluated in this study. Because wild type HBD3 is commercially available, we purchased it at 95% purity (AnaSpec Inc.) The amino acid sequences of the HBD3 and its analogues studied are shown in (Figure 6.1).

Approximately 3 mg Wild-type MccJ25 was obtained by over expression protocol already established by Kaur and colleagues [87]. After purification by flex column the crude MccJ25 was finally purified with the C-18 RP-HPLC, at a flow rate of 2 ml/min 55-80% methanol/water mixture in 55 minutes. Various fractions at different time points were obtained. Fraction containing the desired mass for MccJ25 eluted at 20 minutes on the RP-HPLC column. The calculated mass for MccJ25 was 2107 MW and the mass was determined to be $[M+H]^+$ 2107.7 (Figure 6.2). The elute from desired peak was collected, pooled and lyophilized. The yield or the concentration of MccJ25 was measured using UV-Vis spectrophotometer at 278 nm.

Figure 6.1 Amino acid sequences of HBD3, and the three HBD3 fragments. Substitution of cysteine residues with valine in the fragments is shown in red.

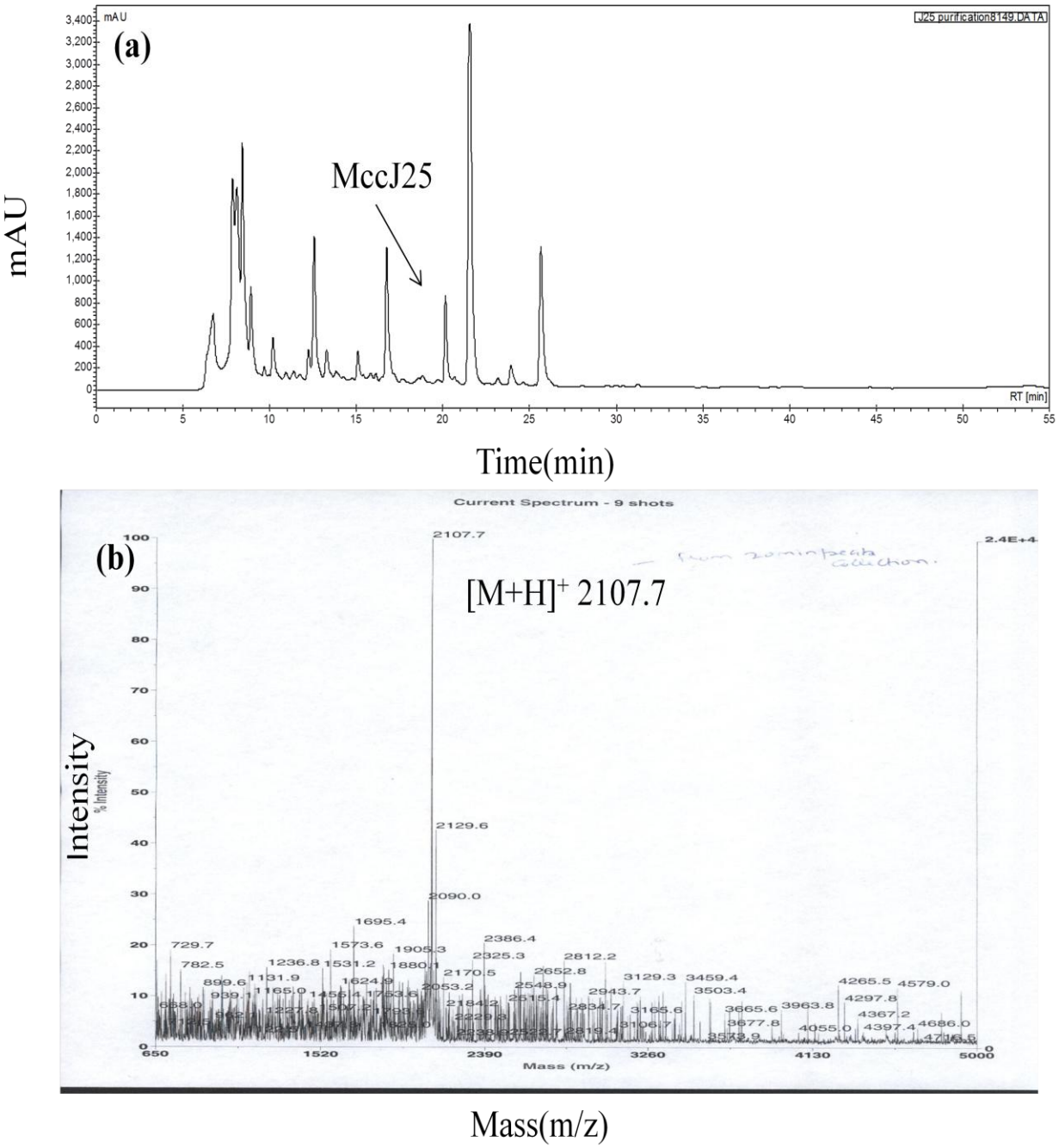


Figure 6.2. a) HPLC chromatogram of crude Microcin J25 (MccJ25) showing elution of MccJ25 at 20 min following acetonitrile/water gradient on a reversed-phase column. mAU is a symbol for the milli-absorbance unit. b) MALDI-TOF of pure MccJ25, purified by reversed-phase HPLC, showing [M+H] + peak (observed 2107.7 and calculated. 2107). Intensity is relative abundance or signal intensity of the ions and m/z is mass to charge ratio.

6.1.1. SPPS Synthesis of HBD3 Analogues

Three C-terminal HBD3 analogues were designed as short fragments of wtHBD3 with different amino acid chain lengths (Figure 6.1). In each analogue, cysteine was replaced with valine to remove di-sulfide linkages and render the fragment linear.

Standard SPPS with Fmoc chemistry was used for the synthesis of the three analogues. Synthesis was carried out in a syringe like reaction vessel with a frit at the bottom. 10AA analogue was synthesized first. Amino acid chain was built on 2-chlorotrityl resin (0.2mmol ~ 127mg) in the reaction vessel. The First amino acid lysine (5eq or 1mmol) lysine was coupled using N, N-Diisopropylethylamine (DIPEA) (1 mmol) which is 5 times the amount of resin. The amount of amino acids added after first amino acid was two equivalents (2eq) of the resin i.e.0.4 mmol. Also, all of the amino acids were added after first lysine was activated in HCTU (0.4mmol)/HoBT (0.4mmol)/NMM (100 μl) mixture in 1-2 ml DMF. The proper peptide chain elongation was ensured with Ninhydrin test after every coupling step and test cleavage after every third amino acid.

After building 10AA amino acid chain, the peptide was cleaved from the solid support. A treatment with 95% TFA, 5% tri-isopropylsilane in water, for 2 hour at room temperature with continuous shaking of the vessel was used to simultaneously de-protect the side chains and cleave the crude peptide from the resin.

On a semi-preparative RP-HPLC column using gradient elution, 22 mg of the crude 10AA analogue was purified. During method development for purification several schemes were tried such as 90-10% acetonitrile/water in 40 minutes at 1ml/min flow rate, with this method desired fraction was found eluting with few other fractions, then a gradient of 50-20% acetonitrile/water in

60 minutes with the flow of 1.3ml/min and finally the used for purification was 10-35% acetonitrile/water in 60 mins with a flow rate of 1.3 mL/min. (Table 6.1)

The successful method also suggests that 10AA analogue was not very hydrophobic as it eluted mostly at gradient with 87% water in the mobile phase. Peaks eluted at 10.9-11.2, 13.4-14.5, 17-18, 26-27, 27-28, and 33-34 minutes were collected. Mass spec, through MALDI-TOF with α-cyano-4-hydroxycinnamic acid (HCCA) as a matrix, for all of the elutions was taken and elution at peak 13.4-14.5 had the correct mass (Figure 6.3a). Calculated mass for 10AA was 1283 and the mass found was $[M+H]^+$ 1282.6. (Figure 6.3b)

Table 6.1. Gradient elution scheme for 10AA and 20AA HBD3 analogues.

Time (min)	Acetonitrile (%)	Water (%)
0	10	90
30	17.5	82.5
60	35	65

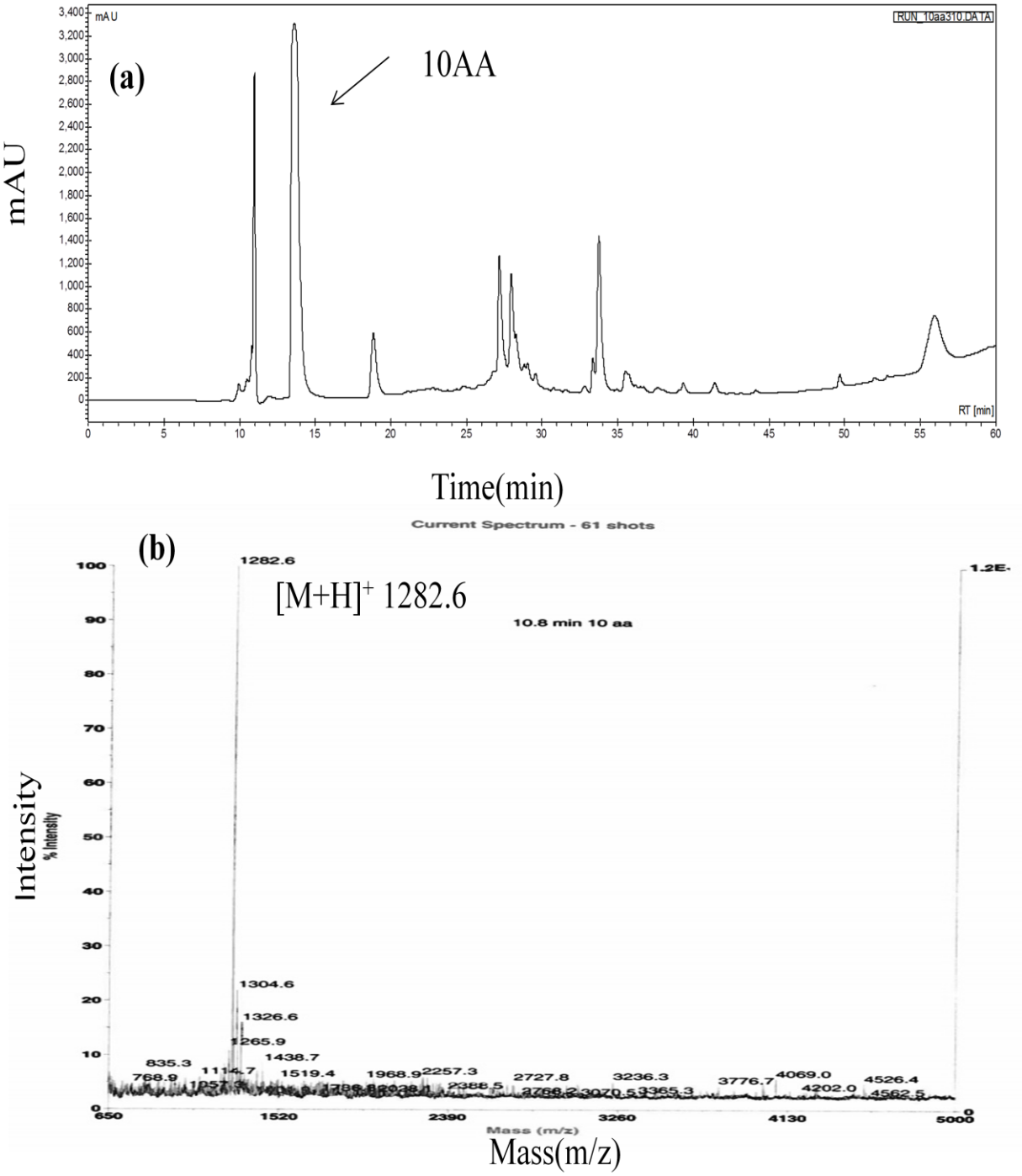


Figure 6.3. a) HPLC chromatogram of crude 10AA analogue showing elution of at 13.1 min following acetonitrile/water gradient on a reversed-phase column. mAU is a symbol for the milli-absorbance unit. b) MALDI-TOF of pure 10AA analogue, purified by reversed-phase HPLC, showing $[M+H]^+$ peak (found 1282.6, calculated. 1283). Intensity is relative abundance or signal intensity of the ions and m/z is mass to charge ratio.

20AA analogue was synthesized in a manner similar to 10AA analogue. Beyond the addition of tenth amino acid, ninhydrin test and test cleavage were performed after every third coupling. Double coupling was performed for the last four amino acids, because of the positive ninhydrin test after single coupling.

The purification scheme used for 20AA analogue was the same as the 10AA analogue. Peaks eluted at 13-14.5, 23-25, 29-30, 35-36.5, and 51.5-53 minutes were collected. Similarly, the mass spectrum, through MALDI-TOF with α- cyano-4-hydroxycinnamic acid (HCCA) as a matrix, for all of the elutions was taken and the correct mass was found in elution at peak 35-36.5 (Figure 6.4). Calculated mass for 10AA was 2384 and the mass found was $[M+H]^+$ 2383.1 (Figure 6.4). However, complete resolution between 19AA analogue and 20AA analogue could not be achieved. Several other schemes were tried to achieve resolution of 20AA analogue from other truncated peptides but best results were achieved with 10-35% acetonitrile/water in 60 minutes with a flow rate of 1.3 mL/minute scheme. Nonetheless, the major component from the eluate was 20AA analogue and 19AA analogue was only a small fraction. Overall 24-28 mg of purified peptide was obtained.

The synthesis of 30AA, linear with all cysteines mutated with valine, was attempted. However, the chain could be elongated only up to 28 AA analogue as identified by mass spec of crude peptide showing a mass of 3386.4 $[M+H]^+$ (Calculated. 3387) (Figure 6.5).

The coupling of glycine moiety to the 28th amino acid arginine could not be achieved even with three consecutive couplings. Since the manual synthesis of such a long amino acid chain is usually fraught with many pitfalls, the elongation of a 28 amino acid chain was a satisfactory result, because the appropriate balance of positive charge and hydrophobicity was attained.

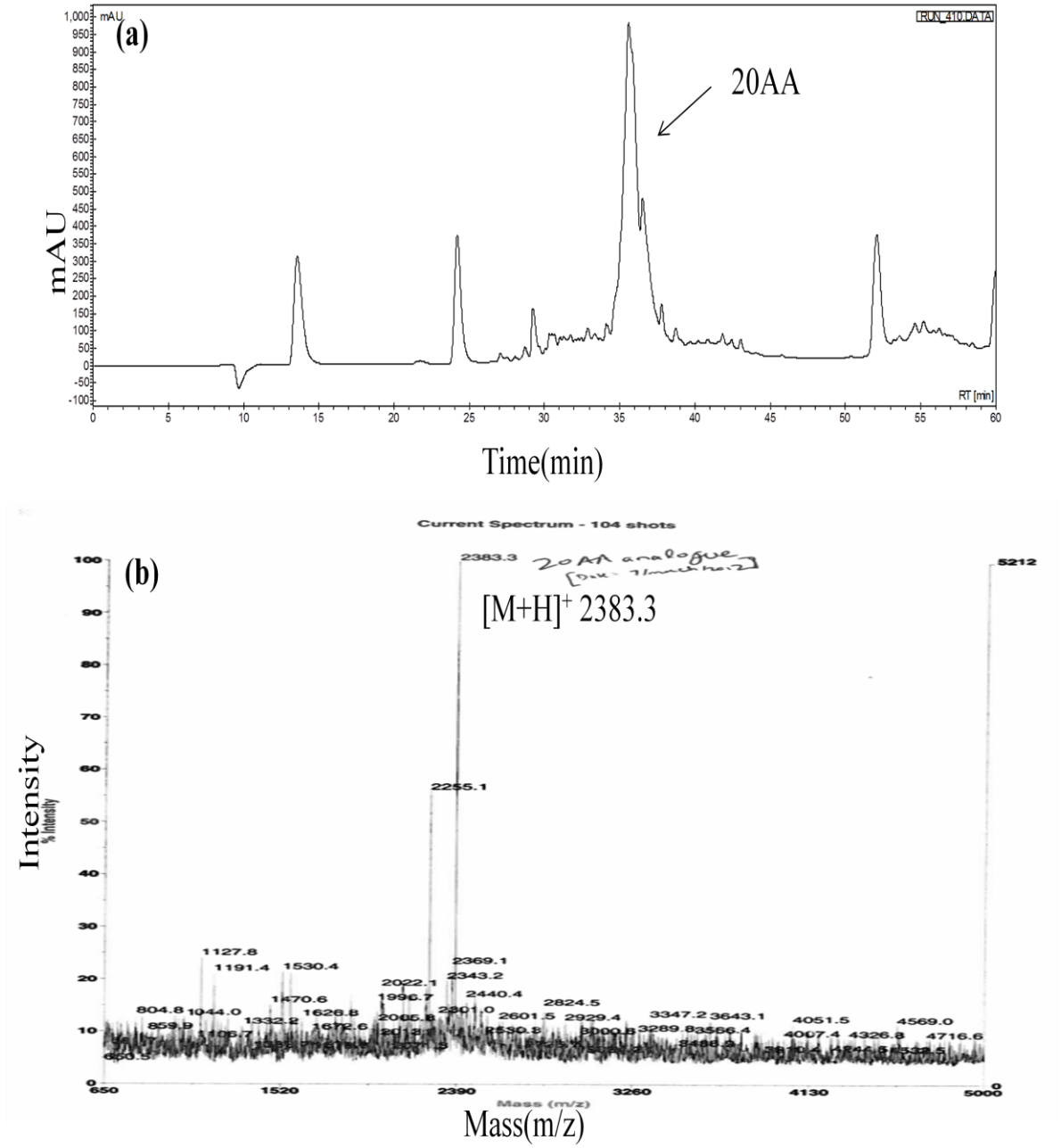


Figure 6.4. a) HPLC chromatogram of crude 20AA analogue showing elution at 35 min following acetonitrile/water gradient on a reversed-phase column. mAU is a symbol for the milli-absorbance unit. b) MALDI-TOF of pure 20AA analogue, purified by reversed-phase HPLC, showing $[M+H]^+$ peak (found 2383.1, calculated. 2384). Intensity is relative abundance or signal intensity of the ions and m/z is mass to charge ratio.

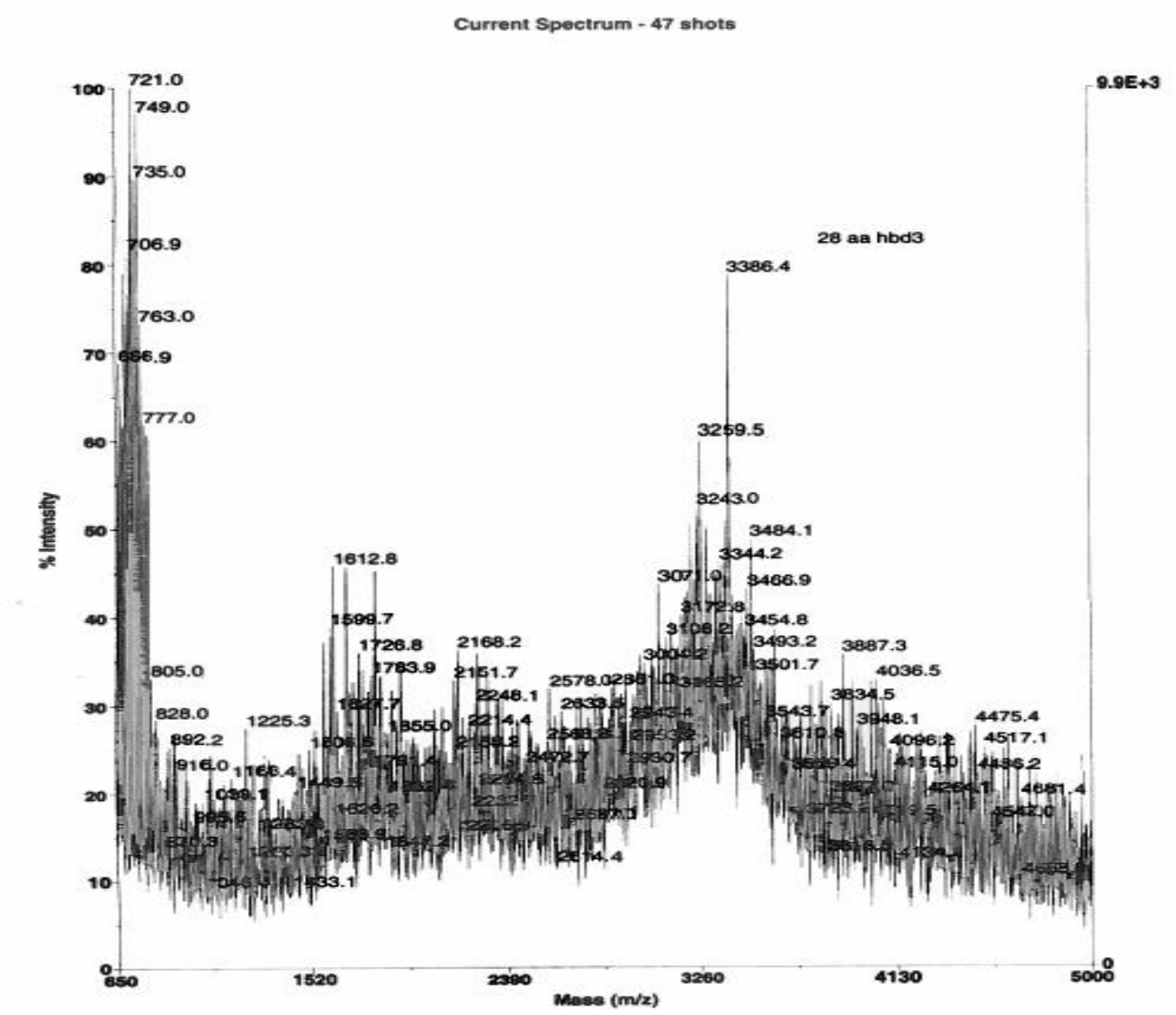


Figure 6.5. : MALDI-TOF of crude 28AA analogue, showing $[M+H]^+$ peak (found 3386.4, calculated. 3387). Intensity is relative abundance or signal intensity of the ions and m/z is mass to charge ratio.

6.2 Microbicidal effect of peptides

Peptides HBD3 its analogues and Mccj25 were assessed for MBC. Minimum bactericidal concentration (MBC) is described as the lowest concentration of each drug that resulted in a 99.9% reduction in CFU of the initial inoculum. All the peptides have shown intermediate to potent killing activity towards *M. haemolytica*. The final inoculum size should be $5x10^5$ cfu/ml, but varied between $1x10^5$ cfu/ml to $1x10^6$ cfu/ml and is acceptable as per M7-A7 guidelines. However, some published studies have taken final inoculum in the range of 10^4 cfu/ml to 10^5 cfu/ml [88].

The comparison of antibacterial activity at different concentrations of HBD3 revealed that HBD3 was equally active at 100.0 µg/ml and 50.0 µg/ml. However, the concentrations 50 µg/ml and 25.0 µg/ml, 25.0 µg/ml and 6.3 µg/ml and 6.3 µg/ml and 3.1 µg/ml were statistically different. The concentrations 3.1 µg/ml and 0.8 µg/ml were not active and there was no statistical difference between them (Table 6.2 and Figure 6.6).

Similar to HBD3, 20AA analogue was equally active at both 50.0 µg/ml and 100.0 µg/ml. However, the concentrations 50.0 µg/ml and 25.0 µg/ml, 25.0 µg/ml and 6.3 µg/ml and 6.3 µg/ml and 3.1 µg/ml were statistically different from each other. Again, concentrations 3.1 µg/ml and 0.8 µg/ml were not active and there was no statistical difference among them (Table 6.3 and Table 6.7).

The activity of 10AA at all the concentrations was similar without any statistical difference. It is worth noting that this is observed only with 10AA analogue which is very likely due to a smaller inoculum size (10^4 cfu/ml) used (Table 6.4 and Figure 6.8).

MccJ25 exhibited microbicidal effect; however the reduction in colony count was much less in comparison to other peptides Different concentrations of MccJ25 were compared for antibacteri-

al activity against *M.haemolytica* but no statistically significant difference was observed (Table 6.5 and Figure 6.9).

The antibacterial effect of 28AA analogue's concentrations 100.0 µg/ml to 6.3 µg/ml was statistically similar as was the antibacterial effect for the concentrations 3.1 µg/ml-0.8 µg/ml. The only difference was observed between 3.1 µg/ml and 6.3 µg/ml (Table 6.6 and Figure 6.10). Figure 6.1 shows a clear dose response when *M.haemolytica* was treated with crude 28AA analogue. The number of colony forming units progressively increased with decreasing peptide concentration. The MBC and LD50 of all the peptides have been compared (Table 6.7). While HBD3 and HBD3 20AA analogues have equal MBC values of 50 µg/ml, MBC value for HBD3 28AA analogue was the lowest at 12.5 µg/ml, and MccJ25 had highest MBC value of 100 µg/ml. Nearly comparable LD50 values do not effectively discriminate different peptides. However, the value of LD50 is apparent in the fact that it shows MccJ25 kills half of the bacterial population at 6.3 µg/ml.

Table 6.2. Number of colony forming units after treatment with HBD3. The tissue culture dishes were inoculated with 50 µl solution from the respective wells and incubated for 24 hour. (This table shows data from one of three separate experiments.)

Well No.	Peptide conc. µg/ml	No. of CFU	Well No.	Peptide conc. µg/ml	No. of CFU
A1	100.0	0	B1	100.0	0
A2	50.0	0	B2	50.0	0
A3	25.0	31	B3	25.0	9
A4	12.5	42	B4	12.5	101
A5	6.3	210	B5	6.3	390
A6	3.1	>400	B6	3.1	>400
A7	1.6	>400	B7	1.6	>400
A8	0.8	>400	B8	0.8	>400
A10	-	0	B10	-	0
A11	-	0	B11	-	0
A12	-	>400	B12	-	>400

Note: The final inoculum in this experiment was $5x10^5$ cfu/ml.

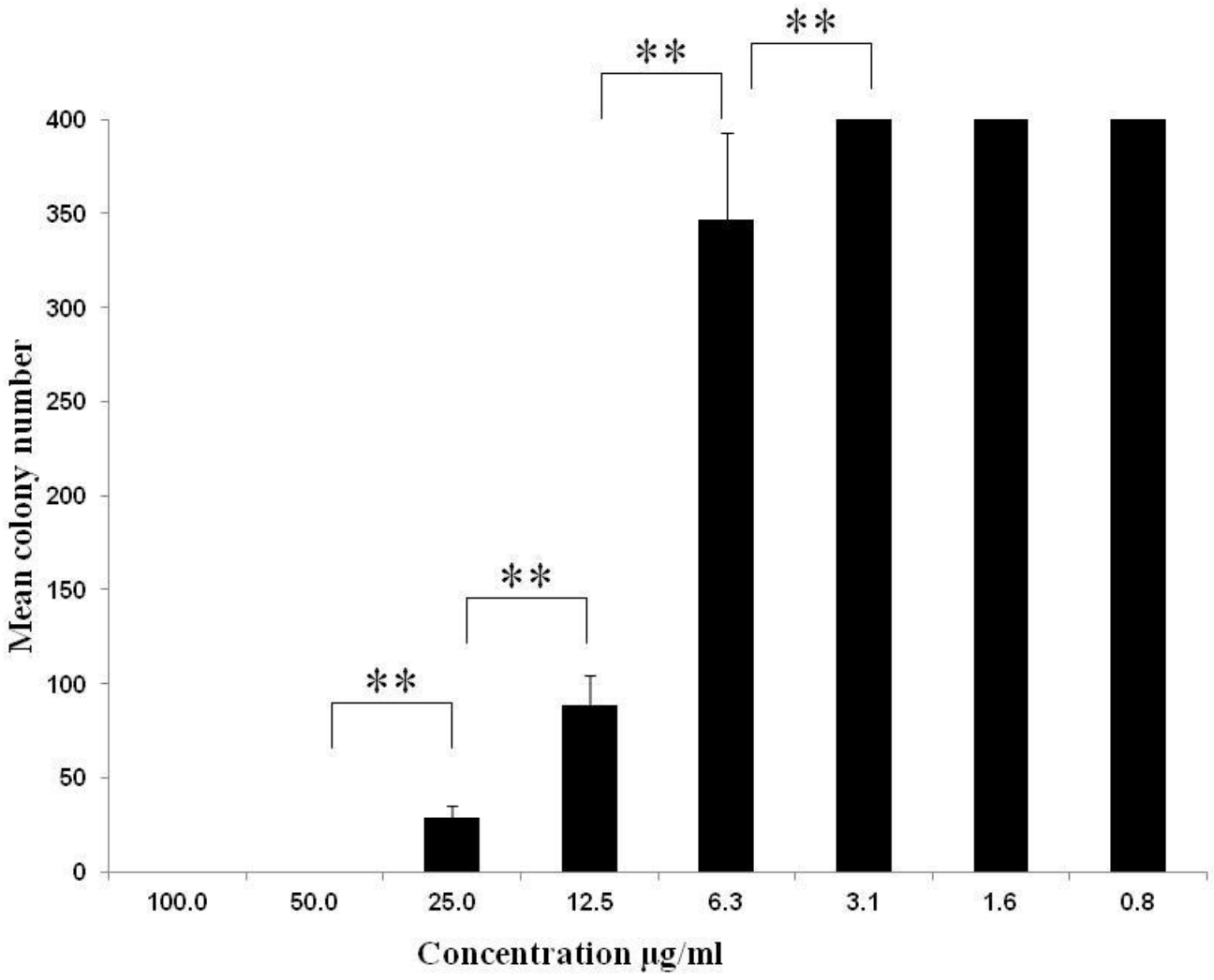


Figure 6.6. Comparisons between microbicidal effects of eight different concentrations of HBD3.

Comparisons were performed using contrasts for example 100.0 µg/ml was compared to 50.0 µg/ml, 25.0 µg/ml compared to 12.5 µg/ml employing non-parametric statistics (ranked data). Statistical significance difference between two concentrations is indicated by **<0.01. The data is represented as Mean±SEM. The statistical difference between 100.0 µg/ml and 50.0 µg/ml concentrations was not significant but the concentration 50.0 µg/ml was statistically different from 25.0 µg/ml (P=0.00).

Table 6.3. Number of colony forming units after treatment with HBD3 20 AA analogue. The tissue culture dishes were inoculated with 50 µl solution from the respective wells and incubated for 24 hour. (This table shows data from one of three separate experiments.)

Well No.	Peptide conc. µg/ml	No. of CFU	Well No	Peptide conc. µg/ml	No. of CFU
A1	100.0	4	B1	100.0	1
A2	50.0	15	B2	50.0	4
A3	25.0	128	B3	25.0	151
A4	12.5	240	B4	12.5	170
A5	6.3	289	B5	6.3	261
A6	3.1	376	B6	3.1	>400
A7	1.6	>400	B7	1.6	>400
A8	0.8	>400	B8	0.8	>400
A10	-	0	B10	-	0
A11	-	0	B11	-	0
A12	-	>400	B12	-	>400

Note: The final inoculum in this experiment was $5x10^5$ cfu/ml.

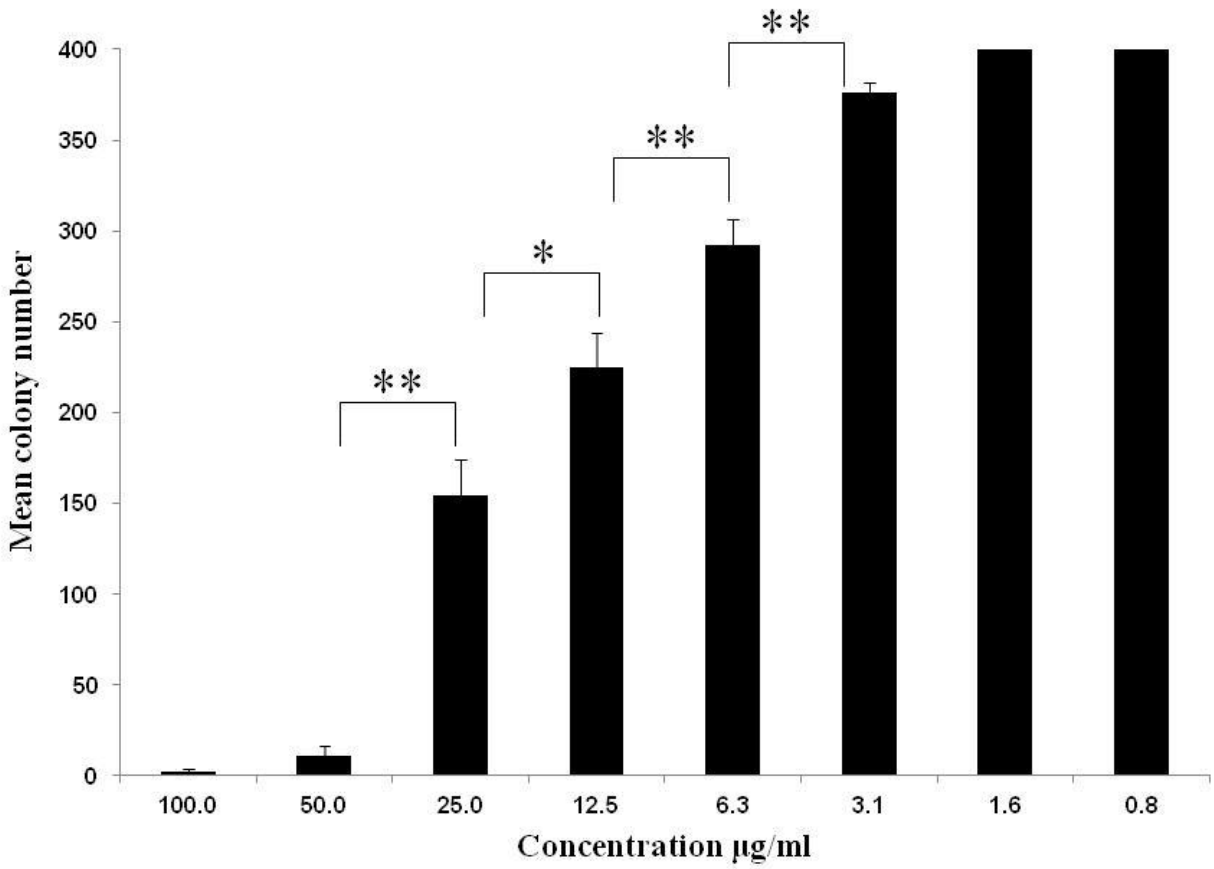


Figure 6.7. Comparisons between microbicidal effects of eight different concentrations of 20AA analogue.

Comparisons were performed using contrasts between adjacent columns. For example 100.0 µg/ml was compared to 50.0 µg/ml, 25.0 µg/ml compared to 12.5 µg/ml employing non-parametric stats (ranked data). Statistical significance difference between two concentrations is indicated by P value*<0.05 and **<0.01. The data are represented as Mean±SEM. The statistical difference between 100.0 µg/ml and 50.0 µg/ml concentration was not significant. But, the concentration 50 µg/ml was statistically different from 25.0 µg/ml (P=0.001). Similarly, 25.0 µg/ml differed statistically from 12.5 µg/ml (P=0.019). P equaled 0.005 (P=0.005) when 12.5 µg/ml and 6.3 µg/ml compared. Also there was a statistical significant difference between conc. 3.1 µg/ml and 6.3 µg/ml (P=0.001)

Table 6.4. Number of colony forming units after treatment with HBD3 10 AA analogue. The tissue culture dishes were inoculated with 50 µl solution from the respective wells and incubated for 24 hour. (This table shows data from one of three separate experiments.)

Well No.	Peptide conc. µg/ml	No. of CFU	Well No.	Peptide conc. µg/ml	No. of CFU
A1	100.0	0	B1	100.0	0
A2	50.0	4	B2	50.0	1
A3	25.0	4	B3	25.0	3
A4	12.5	4	B4	12.5	6
A5	6.3	4	B5	6.3	1
A6	3.1	7	B6	3.1	2
A7	1.6	-	B7	1.6	-
A8	0.8	-	B8	0.8	-
A10	-	0	B10	-	0
A11	-	0	B11	-	0
A12	-	>400	B12	-	>400

Note: The final inoculum in this experiment was 10^4 cfu/ml.

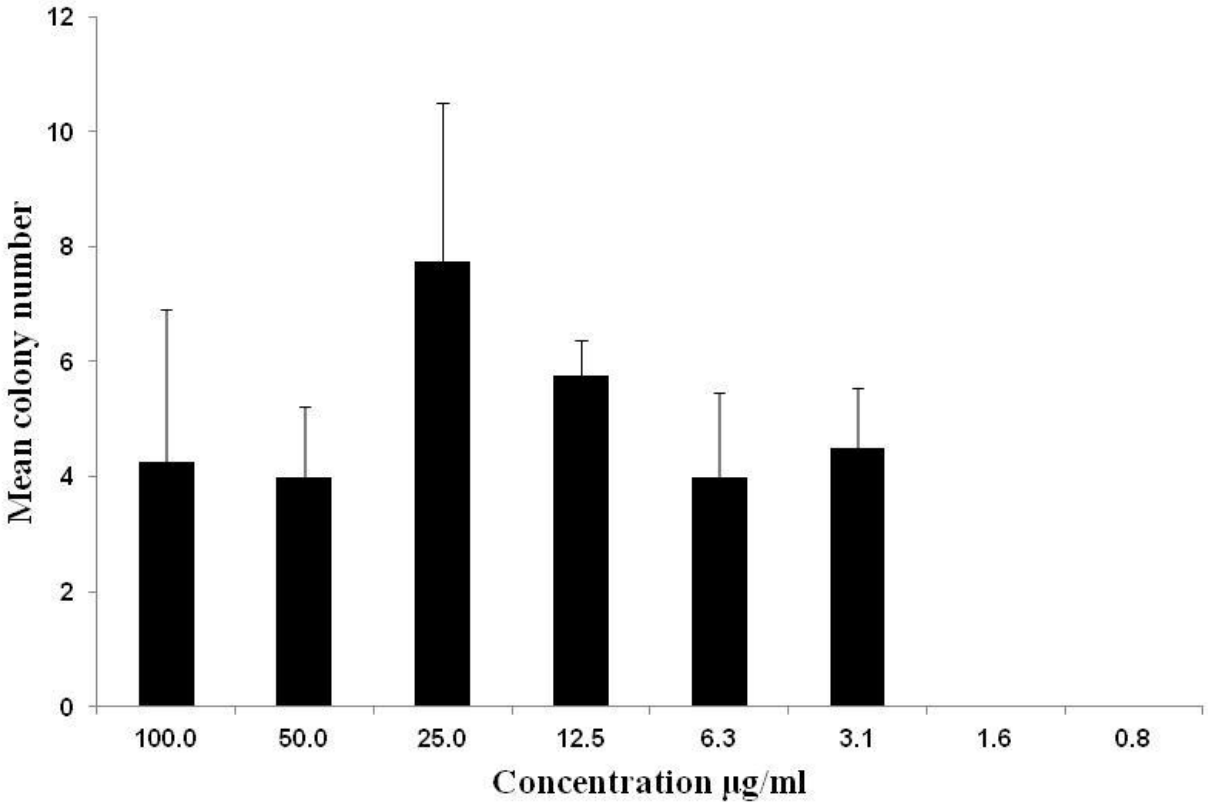


Figure 6.8. Comparisons between microbicidal effects of eight different concentrations of 10AA analogue.

The concentrations were compared using contrasts. For example 100.0 µg/ml was compared to 50.0 µg/ml, 25.0 µg/ml compared to 12.5 µg/ml employing non-parametric stats (ranked data). The data is represented as Mean±SEM. No statistical difference between concentrations was observed.

Table 6.5. Number of colony forming units after treatment with Mccj25. The tissue culture dishes were inoculated with 50 µl solution from the respective wells and incubated for 24 hour. (This table shows data from one of three separate experiments.)

Well No.	Peptide conc. µg/ml	No. of CFU	Well No.	Peptide conc. µg/ml	No. of CFU
A1	100.0	211	B1	100.0	275
A2	50.0	328	B2	50.0	230
A3	25.0	314	B3	25.0	310
A4	12.5	211	B4	12.5	253
A5	6.3	289	B5	6.3	261
A6	3.1	>400	B6	3.1	>400
A7	1.6	>400	B7	1.6	>400
A8	0.8	>400	B8	0.8	>400
A10	-	0	B10	-	0
A11	-	0	B11	-	0
A12	-	>400	B12	-	>400

Note: The final inoculum in this experiment was $5x10^5$ cfu/ml.

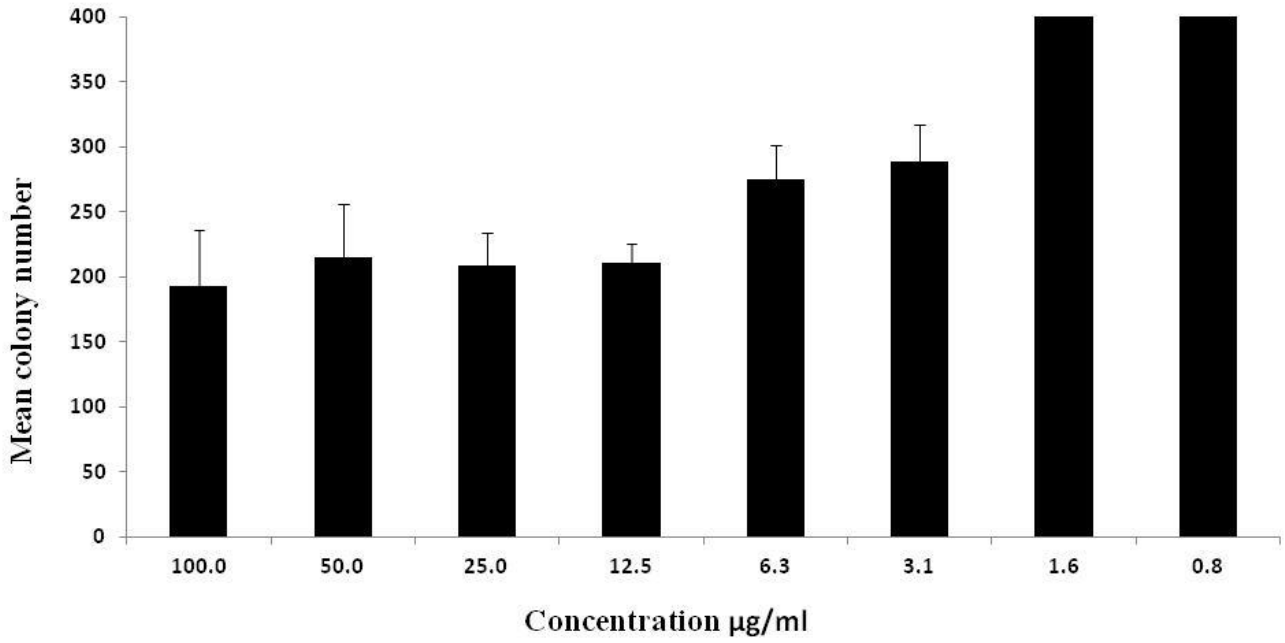


Figure 6.9. Comparisons between microbicidal effects of eight different concentrations of MccJ25.

Comparisons were done using contrasts. For example 100.0 µg/ml was compared to 50.0 µg/ml, 25.0 µg/ml compared to 12.5 µg/ml employing non-parametric stats (ranked data). The data are represented as Mean±SEM. No statistical difference was observed in antimicrobial activity between concentrations.

Table 6.6. Number of colony forming units after treatment with HBD3 28 AA analogue. The tissue culture dishes were inoculated with 50 µl solution from the respective wells and incubated for 24 hour. (This table shows data from one of three separate experiments.)

Well No.	Peptide conc. µg/ml	No. of CFU	Well No.	Peptide conc. µg/ml	No. of CFU
A1	100.0	0	B1	100.0	0
A2	50.0	0	B2	50.0	0
A3	25.0	10	B3	25.0	2
A4	12.5	9	B4	12.5	22
A5	6.3	56	B5	6.3	64
A6	3.1	223	B6	3.1	142
A7	1.6	>400	B7	1.6	>400
A8	0.8	>400	B8	0.8	>400
A10	-	0	B10	-	0
A11	-	0	B11	-	0
A12	-	>400	B12	-	>400

Note: The final size of inoculum was $2x10^5$ cfu/ml in this experiment.

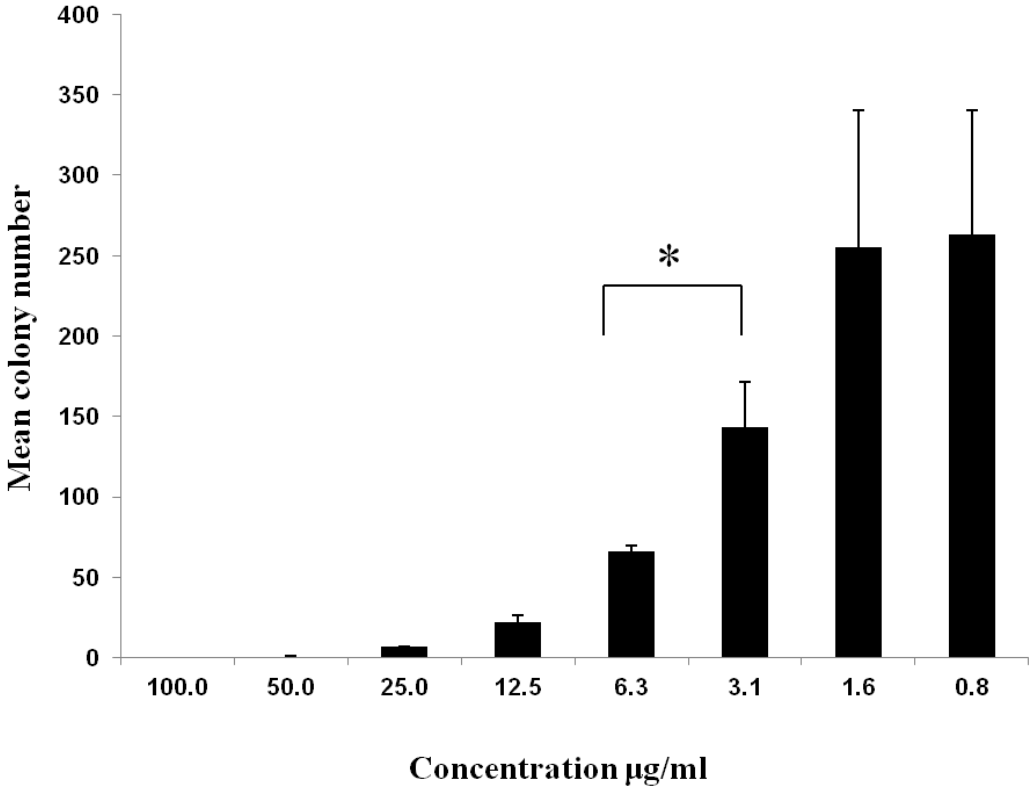


Figure 6.10. Comparisons between microbicidal effects of eight different concentrations of 28AA analogue.

Comparisons were done using contrasts. For example 100.0 µg/ml was compared to 50.0 µg/ml, 25.0 µg/ml compared to 12.5 µg/ml employing non-parametric stats (ranked data). Statistical significance difference between two concentrations is indicated by *P<0.05. The data is represented as Mean±SEM. Only two concentrations 25.0 µg/ml and 12.5 µg/ml differed statistically from each other (P<0.012).

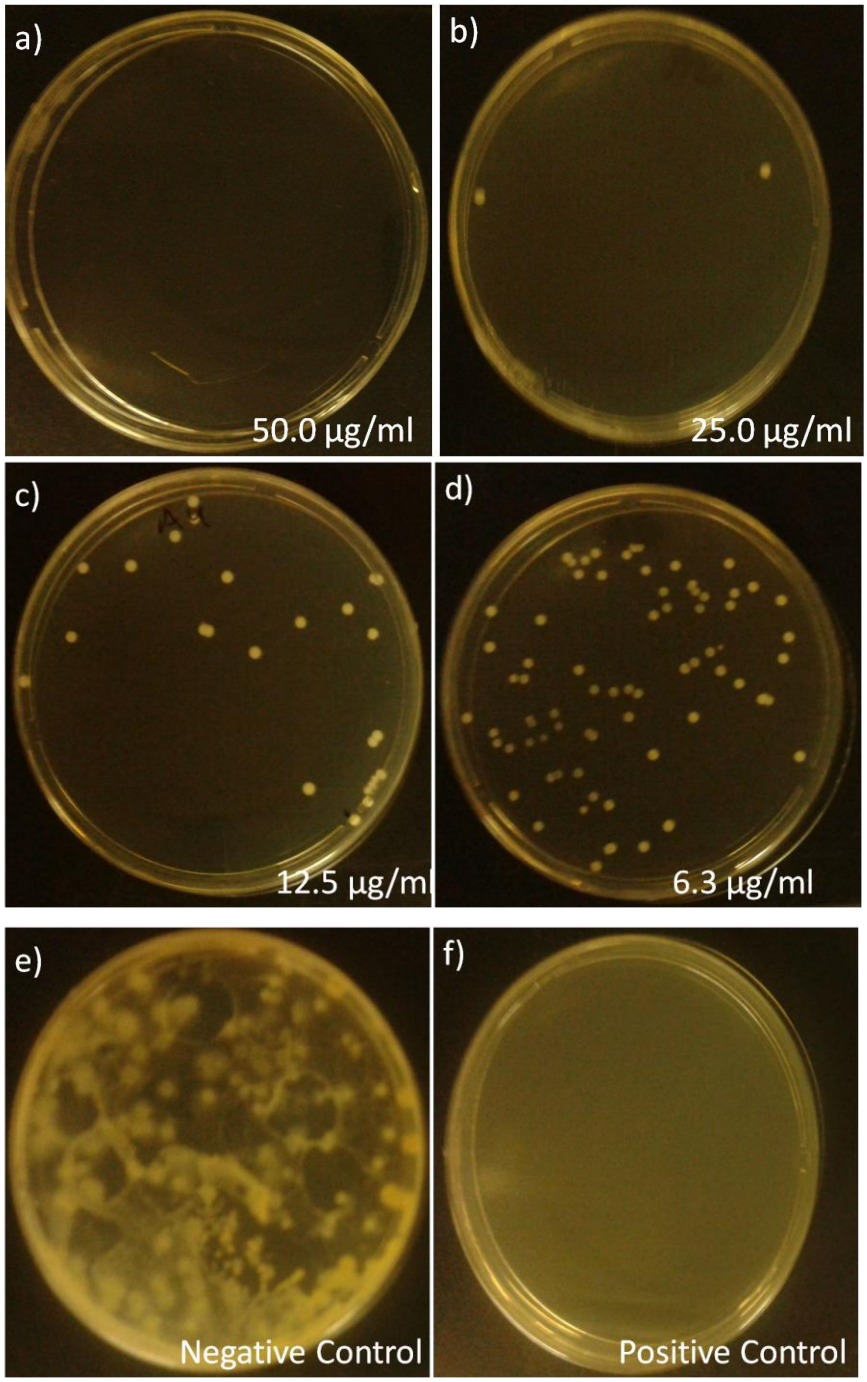


Figure 6.11. Colony forming units of *M. haemolytica*; that survived after incubation with 28AA analogue (a) at concentration 50.0 µg/ml (b) 25.0 µg/ml (c) 12.5 µg/ml d) 12.5 µg/ml and (d) 6.3 µg/ml (e) Negative control plate and (f) positive control plate.

Table 6.7. Comparisons of MBC and LD50 of the peptides. MBC (Minimum Bactericidal Concentration) is the concentration of antibiotic at which 99.9% of the CFU in the final inoculum are killed and LD50 is the lethal dose for ≥ 50% of bacteria.

PEPTIDE	**MBC (µg/ml)**	**LD50 (µg/ml)**
HBD3 10AA analogue	6.3	≥3.1
HBD3 20AA analogue	50.0	3.1
HBD3 28AA analogue	12.5	3.1
HBD3	50.0	6.3
MccJ25	>100.0	6.3

6.2. Chemotaxis Assay

HBD3 is chemotactic for human neutrophils [67]. The chemotaxis of bovine neutrophils in response to HBD3, HBD3 20AA analogue, and HBD3 28AA analogue at 50.0 μg/ml was studied. Neutrophil migration in response to HBD3 and 20AA analogue was found to be statistically significant when compared with PBS (Negative control), but the difference between PBS and 28AA was not significant (Figure 6.12). The result shows that HBD3 and HBD3 20AA analogue were chemotactic for bovine neutrophils at 50.0 μg/ml where as 28AA analogue was not.

6.3. Migration Inhibition Assay

Bovine neutrophils were treated with 50.0 μg/ml of HBD3, HBD3 20AA analogue, and HBD3 28AA analogue and the effect of peptides on neutrophil migration towards fMLP was studied. Neutrophil migration towards fMLP when neutrophils were incubated with HBD3, HBD3 20AA analogue, and HBD3 28AA analogues was statistically different from the PBS, which shows that none of the peptides inhibited migration of neutrophils towards fMLP (Figure 6.13).

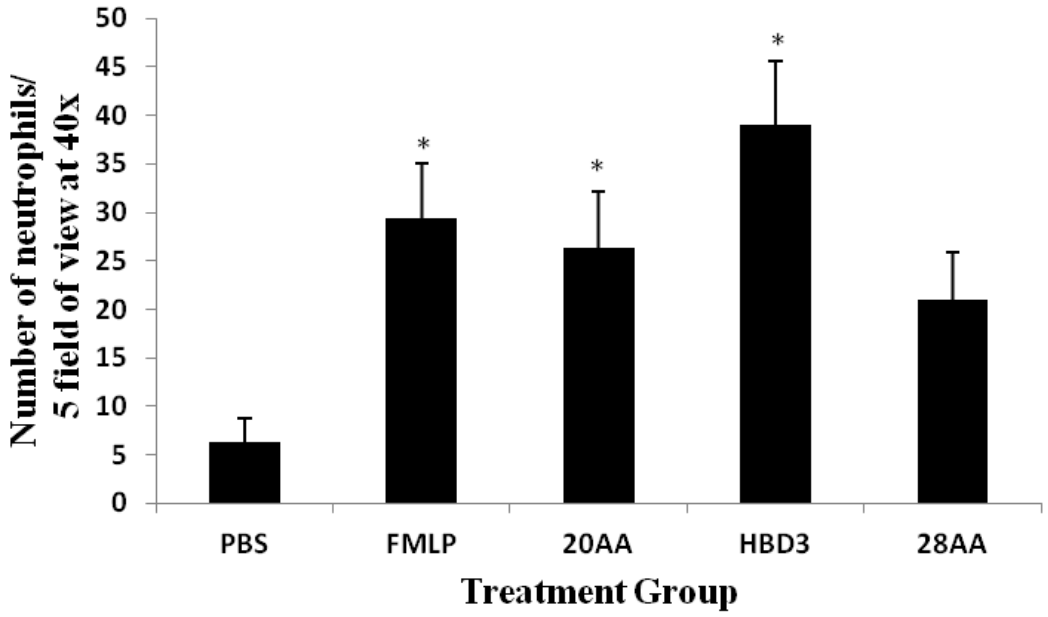


Figure 6.12. Chemotaxis assay of the peptides against bovine neutrophils.

Number of neutrophils that migrated in response to HBD3, HBD3 20AA analogue and, HBD3 28AA analogue after 20 min incubation at 37^0C. The three peptides were used at a concentration of 50.0 μg/ml. fMLP is the positive control. Mean±SEM of three independent experiments. One way ANOVA was followed by Dunnett's multiple comparison test ($P \leq 0.0036$). Asterisks show significant difference from PBS (Negative control) *$P<0.05$.

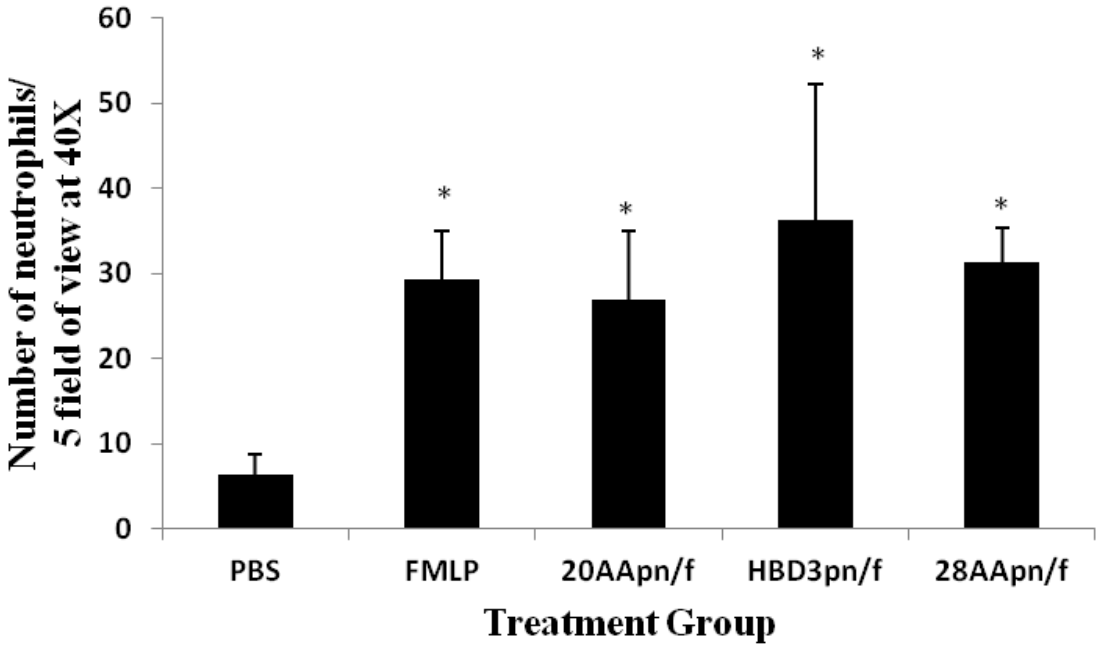


Figure 6.13 Effect of peptides on neutrophil migration towards fMLP.

Number of neutrophils that migrated towards fMLP (114nM) after 20 min incubation at 37^0C. Neutrophils were treated with 50.0 μg/ml HBD3, HBD3 20AA analogue and, HBD3 28AA analogue. pn/f stands for peptide and neutrophils together in the upper well and fmlp in the bottom during 20 min incubation. fMLP is the positive control. Mean±SEM of three independent experiments. One way ANOVA was followed by Dunnett's multiple comparison test ($P \leq 0.0036$). Asterisks show significant difference from PBS (Negative control) *$P<0.05$.

7. Discussion

Our findings indicate that *M. haemolytica* is intermediately sensitive to HBD3 and its analogues. *M. haemolytica* shows resistance or at least intermediately sensitive to MccJ25. This classification of peptide potency is arbitrary, "a strain was defined as sensitive to the peptide if MBC levels were <10 μg/ml, as intermediately sensitive if MBC levels were 10 to 100 μg/ml, or as resistant at MBC levels of >100 μg/ml" [88]. To our knowledge, this is the first study to test the killing and inhibitory effects of HBD3 and MccJ25 against *M. haemolytica*. Both peptides showed killing activity toward *M. haemolytica* as indicated by MBC and LD50 data, but peptides did not show inhibitory effect as seen in MIC data.

In vitro susceptibility tests for cationic peptides are challenging to perfom because polycationic peptides tend to precipitate and bind to the anionic surface of bacterial cells and plastic surface. There are data on the comparison of two methods for evaluating the *in vitro* antimicrobial activities of cationic AMP's [89]. In the first method antimicrobial activity was evaluated according to the National Committee for Clinical Laboratory Standards (NCCLS) protocol using Polystyrene 96-well plates that were incubated for 18 hours at 35°C in air [89]. The second method used was developed by R. E. W. Hancock for testing antimicrobial peptides where-in the Polypropylene 96-well plates were incubated for 18 hours at 37°C in air. The comparative data showed that NCCLS protocol gave MICs and MBCs values that were four times higher than the method proposed by Hancock [89].

Polypropylene 96-well plates were used for antimicrobial evaluation to reduce the possibility of false higher MBCs and MICs as apparent in the results of Giacometti and the results of our preliminary MIC experiment (data not shown). But, we used the NCCLS definition of MBC which describes the "minimal bactericidal concentration (MBC) as the lowest concentration of each

drug that resulted in a 99.9% reduction in CFU of the initial inoculum". The R. E. W. Hancock considered the MBC as the "lowest concentration of each drug that prevented any residual colony formation".

We also carried out the minimum inhibitory concentration (MIC) assay (data not shown) using polypropylene 96-well plates. The only difference between the MBC assay and MIC assay was that after the bacteria in the wells were incubated with 50 µl of different concentrations of peptides and incubated for 2 hours, the 50 µl BHI broth was put on the top of each well and then incubated for another 18 hours.

None of our five peptides inhibited visible growth of the *M. haemolytica* when compared with the positive control. The bactericidal nature of the cationic peptides could be the possible explanation of these results. Ampicillin at a concentration of 24 µg/ml served as negative control in our experiments and there was no growth in the negative wells. Since there were 10000-25000 bacteria/well and even if 0.1% of them survived and the peptide got consumed or deactivated over next few hours and media was still available for their growth. It is very likely that bacteria resumed growth to give false negatives. To ascertain the real cause we need to do time-kill studies.

7.1. Peptide Selection, Synthesis and Antimicrobial evaluation.

HBD3 and MccJ25 were selected as potential candidates. Wild-type HBD3 with purity ≥ 95% was obtained from AnaSpec Inc. The data contained in this thesis show synthesis and characterization of MccJ25 and three analogues of HBD3. We planned to synthesize HBD3 analogues namely 10AA (RGRKVVRRKK), 20AA (KEEQIGKVSTRGRKVVRRKK) & 30AA (GRVAVLSVLPKEEQIGKVSTRGRKV VRRKK), using Fmoc Solid Phase Peptide Synthesis.

The 10AA and 20AA analogue of HBD3 were synthesized successfully, whereas 30AA analogue could not be synthesized. Peptide showed correct mass ($M+H^+$ found 3386.4, calculated. 3387) up to 28 amino acids during the test cleavage. However, addition of the last two amino acids followed by test cleavage did not show the desired mass. Amino acid chain could be elongated up to only 28AA, as confirmed by MALDI-TOF even after repeated coupling. Mass spec. of 28AA crude peptide shows couple of other peaks along with 28AA analogue peak suggesting impurities in the crude peptide. The crude peptides (MccJ25, 10AA and 20AA analogue) were purified by RP-HPLC and then by MALDI-TOF mass spectrometry to confirm mass of the crude and pure peptides.

7.1.1. MccJ25

MccJ25 kills bacteria by inhibiting the RNA Polymerase (RNAP) but this microcin peptide has to enter the bacterial cell to accomplish this action. The MccJ5 sensitive strains have the transporter proteins such as outer-membrane protein FhuA and the inner membrane protein SbmA to facilitate the transport of the antimicrobial peptides into the cells [90, 91]. It could be very likely that *M. haemolytica* does not possess such membrane proteins. Furthermore MccJ25 is resistant to many bacteria of the family *Enterobacteriaceae* that are not related to the MccJ25 producing members [92]. It could be argued that MccJ25 shows a narrow action spectrum activity *in vitro*.

However, MccJ25 could be active *in vivo* against resistant species [92, 93]. It has been shown that resistant bacteria become susceptible to antibiotics upon their entry into phagolysosomes of macrophages. Most likely the low pH environment in the lysosomes of the macrophages leads to the nonspecific MccJ25 uptake into the bacterial cell through altered bacterial membrane permeability. Also, MccJ25 has been shown to cause disruption of the membrane potential [93] and

hence it can be speculated that inside a macrophage *M.haemolytica* could become sensitive to MccJ25.

7.1.2. HBD3

In our study, HBD3 was very effective in killing *M. haemolytica* with MBC value of 50 μg/ml. The testing of the bactericidal activity of the HBD3 against 28 species and 55 strains of Gram-positive cocci and Gram-negative fermentative and nonfermentative rods showed it be intermediately or highly effective [88]. However, MBC value in our study is higher when compared to the study done by Hany Sahly and colleagues in which only 3 strains out of 55 tested showed intermediate susceptibility (MBC value of 50.0 μg/ml), whereas rest of the strains were highly sensitive to HBD3 with MBC's ranging from 0.1 to 6.3 μg/ml [88]. The reason for lower MBC's in their study could be attributed to use of a lower final bacterial inoculum of 10^4 to 10^5/ml [88], compared 10^5 to 10^6/ml used in our study. This phenomenon of lower MBC corresponding with low final inoculum size was also observed in our study with 10AA analogue. We tested various concentrations of this analogue with final inoculum size of 10^4 cells/ml of *Mannehemia haemolytica* and this gave us MBC of 6.3 μg/ml.

7.1.3. HBD3 Analogues

HBD3 analogues showed intermediate activity against our bacteria of interest. 20AA analogue was active against *M. haemolytica* in the same concentration as the wild type HBD3, after averaging results from three different experiments, in duplicates, they showed equivalent MBC values.

20 AA analogue's potent activity can be attributed to its high positive charge (+9). HBD has a higher positive charge of +11 but showed anti-microbial activity similar to that of 20AA ana-

logue. Therefore, it will be interesting to replace a couple of positively charged basic polar amino acids such as arginine or lysine with valine, a neutral nonpolar amino acid, to the analogue more hydrophobic and increasingly selective for bacterial membranes. This argument is further supported by the fact that 10 AA analogue with +7 charge displayed excellent activity against *M. haemolytica* with 6.3 μg/ml as MBC when used with a small inoculum. The testing of this analogue with a higher bacterial inoculum, similar to other peptides, would have further bolstered our results. Nevertheless, MBC of 10AA analogue was lower than both HBD3 and 20AA analogue and therefore we may be able extrapolate this result for higher inoculum size.

Zhou and Liu made eight C-terminal 10AA analogues of HBD3 replacing cysteines with either Valine, Tryptophan or Tyrosine. Out of these, Valine was easier to build via SPPS synthesis and was found to be most active [66]. They also made a dimer of Valine 10AA analogue, which turned out to be the most potent analogue. Instead of making a dimer of 10AA analogue, we chose to elongate the peptide chain up to 20 and 28 AA with mutation of all Cysteines with Valines. Both 20AA and 28AA analogues were active with 28AA peptide showing better activity than wild-type HBD3 [66]. These researchers further tested their peptides against *Pseudomonas aeruginosa*, a Gram –ve bacteria, in the same concentration range as ours. Comparing their study with ours, we found that the 10AA analogue had similar MBC values (50.0 μg/ml) for both *Pseudomonas aeruginosa* and *M. haemolytica*. However, their dimer was more potent than our 20AA analogue.

Studies have shown that some "C-terminal (R36-K45) analogues of human β-defensin-3 non-covalently dimerize and adopt a well-defined structure in the aqueous solution and on the lipid bilayer" [66, 94]. Elucidating the underlying physico-chemical properties was beyond the scope of this study. However, we can speculate about the 3D structure of 20AA analogue based on the

nearly comparable MBC results of 20AA analogue with wtHBD3, and findings of the previous studies performed with NMR, fluorescence correlation spectroscopy and molecular dynamics simulation techniques [94].

It is possible that the 20AA analogue, dimerized both in aqueous solution and on the membrane surfaces, which tend to localize the positive charge density. Hence, it could be that the compactness of positive charge and the growth of well defined structure once in solution accounts for the bactericidal efficacy of the 20AA analogue. Alternatively, the 20AA analogue would just remain as a linear monomer and its potent activity results from increased flexibility due to a loss of secondary structure. At this point we can not speculate about the structural conformation of 28AA analogue, since it was used in the crude form. Its activity could be a result of a truncated peptide.

7.2. Chemotaxis and migration inhibition assay

Anti-microbial peptides may exert a killing action against the microbes and they also may have a range of immunomodulatory functions such as modulating recruitment of neutrophils [64]. Defensins have also previously been studied for their chemotactic properties. Niyonsaba and colleagues tested the chemotaxis of human neutrophils towards HBD2 and HBD1 and found HBD2 to be chemotactic [90]. To find out whether bovine neutrophils migrate in response to HBD3, 20AA and 28AA analogue, we conducted chemotaxis assay using Boyden's chamber.

Similar to HBD2, the HBD3 and its 20AA analogue were found to be chemotactic in our experiments. Previous data have shown that HBD2 was chemotactic only when the human neutrophils were pretreated with TNF-α [90]. Untreated neutrophils would not migrate in response to HBD-2. But, in our study, we used only untreated bovine neutrophils and there was a significant migration in response to HBD3 and 20AA analogue when compared to the negative control

(P=0.0036). It could be a species specific phenomenon. Furthermore, we used the peptides at 50.0 μg/ml and compared to concentrations of 0-10 μg/ml in previous studies [95]. It was earlier reported that cysteine mutated analogues of HBD3 might lose their chemotactic activity [67], but here 20AA analogue's chemotactic behavior could be attributed to our use of higher concentration (50.0 μg/ml). To elucidate the chemotactic behavior of 20AA analogue it must be tested against a wide concentration range; neutrophils from other species should also be tested.

It might be possible that bovine neutrophils are generally responsive to the Cysteine mutated HBD3 analogues. The data by Taylor and colleagues demonstrated the indispensability of three cysteine disulfide bridges for chemotactic activity [96]. They showed that HBD3 analogues lacking disulfide bridges retained chemoattractant capability. HBD3 analogues which had cysteines replaced with alanines was devoid of any chemotactic activity. However, the chemotactic activity was not lost when the fifth cysteine was retained and other five were replaced. These anomalous results could be due to variation in environmental conditions or different cell species used [96].

Therefore, in the light of the new evidence it seems essential to do more studies to understand the relationship between structure and function of the HBD3 and its linear analogues with their chemotactic property. The chemotaxis in 28AA analogue well and PBS well was statistically not different. This anomaly can be accounted for if we take in to consideration that 28AA was in crude form and had contained a few truncated peptides. Lastly, the treatment of neutrophils with any of peptides did not affect their migration towards fMLP.

8. LIMITATIONS OF THE STUDY

I could not fully characterize the 28AA analogue and its activity could be the result of many truncated peptides. The use of manual SPSS process to synthesize the peptides in this study added significant amount of time to the work because automated peptide synthesizer for SPPS in our lab was out of order. This extra time could have been used for further anti-microbial and biological characterization of the peptides.

9. FUTURE STUDIES

It will be useful to study the phagocytic activity of the immune cells such as neutrophils and macrophages that have been exposed to the anti-microbial peptides characterized in this study. Furthermore, the peptides should be evaluated against additional bacteria such as *Histophilus somni* that cause respiratory diseases in domestic animal species. There also is a need to determine whether the peptides affect the life-span or production of anti-microbial molecules such as reactive oxygen species or even affect their ability to migrate into diseased tissues; the later of course requires *in vivo* studies.

10. CONCLUSIONS

Three analogues of HBD3 were successfully synthesized and characterized. HBD3 and its 3 analogues are active against *M. haemolytica*, but MccJ25 proved not very efficacious. HBD3 is chemotactic for bovine neutrophils at 50.0 µg/ml. HBD3 and 20AA did not inhibit migration of neutrophils.

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